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REVIEW ARTICLE

A critical analysis of current *in vitro* and *in vivo* angiogenesis assays

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Summary

The study of angiogenesis has grown exponentially over the past 40 years with the recognition that angiogenesis is essential for numerous pathologies and, more recently, with the advent of successful drugs to inhibit angiogenesis in tumours. The main problem with angiogenesis research remains the choice of appropriate assays to evaluate the efficacy of potential new drugs and to identify potential targets within the angiogenic process. This selection is made more complex by the recognition that heterogeneity occurs, not only within the endothelial cells themselves, but also within the specific microenvironment to be studied. Thus, it is essential to choose the assay conditions and cell types that most closely resemble the angiogenic disease being studied. This is especially important when aiming to translate data from *in vitro* to *in vivo* and from preclinical to the clinic. Here we critically review and highlight recent advances in the principle assays in common use including those for endothelial cell proliferation, migration, differentiation and co-culture with fibroblasts and mural cells *in vitro*, vessel outgrowth from organ cultures and *in vivo* assays such as chick chorioallantoic membrane (CAM), zebrafish, sponge implantation, corneal, dorsal air sac, chamber and tumour angiogenesis models. Finally, we briefly discuss the direction likely to be taken in future studies, which include the use of increasingly sophisticated imaging analysis systems for data acquisition.

Keywords
angiogenesis, differentiation, endothelial, migration, tumour

Introduction

Angiogenesis, the development of new blood vessels from the existing vasculature, is essential in normal developmental processes and is a hallmark of over 50 different disease states including cancer, rheumatoid arthritis and psoriasis (Carmeliet & Jain 2000). Physiological angiogenesis is a highly organized sequence of cellular events comprising vascular initiation, formation, maturation, remodelling and regression, which are controlled and modulated to meet the tissue requirements. In contrast, pathological angiogenesis is less well controlled and although the initiation and formation stages occur, the vessels rarely mature, remodel or regress in disease. Therefore, improving our understanding of the factors and processes involved in the maturation, remodelling and regression of vessels is necessary for the

treatment of angiogenesis-dependent disease states. Moreover, the use of 'angiogenesis' as a generic term to describe vascular proliferation suggests a single process whereas in reality angiogenesis is different in conditions such as tumours, eye disease or inflammation and these diverse conditions do not produce a homogenous population of new blood vessels (Carmeliet & Jain 2000). Therefore, there is a need to study angiogenesis in greater detail and in the relevant disease and tissue to allow the development of new/improved therapeutic strategies.

The main technical challenge in any study of angiogenesis is the selection of the most appropriate assay. The ideal angiogenesis assay would be robust, rapid, reproducible with reliable readouts, automated computational analysis, multi-parameter assessment, including positive and negative controls and should relate directly to results seen in the clinic. Despite the increasing numbers of both *in vitro* and *in vivo* assays, a 'gold-standard' angiogenesis assay has yet to be developed; therefore, a combination of assays are required to identify the full range of effects of a test protein or to identify the molecular and/or cellular events in angiogenesis.

Although a majority of *in vitro* assays focus on endothelial cells, the migration, proliferation and differentiation of which are central to the angiogenic process, other cell types are important. Therefore, the most translatable assays would include the supporting cells (e.g. smooth muscle cells, pericytes, fibroblasts and tumour cells), the extracellular matrix (ECM) and/or basement membrane and circulating blood. However, despite various attempts at co-culturing endothelial cells with different cell types, no *in vitro* assay currently exists which satisfactorily models all the components of this complex process. Moreover, although *in vivo* this complexity exists, the assays are limited by the species used, organ sites, administration of the test substances and lack of quantitative analysis. This review therefore critically appraises the *in vitro* and *in vivo* assays currently in use and discusses the direction for future studies.

In vitro assays of angiogenesis

Endothelial cells in vitro

In adults, most endothelial cells are quiescent and poised for repair, and whilst it was originally thought that all endothelial cells were homogeneous, it is now commonly accepted that there is a high degree of heterogeneity along the vascular tree, to allow biological adaptation to local needs. At the morphological level, vessel phenotype can be described as continuous, where endothelial cells line the complete internal surface of the vessel wall; fenestrated, where endothelial cells are permeable because of the presence of small openings (fenestrae); and sinusoidal, which have large lumen, many fenestrations and a discontinuous (or absent) basal lamina. Apart from this traditional classification, there are also differences in endothelial size, shape, complexity of junctions and presence or absence of plasmalemmal bodies. The functional heterogeneity of endothelial cells also needs to be taken into consideration, including roles in control of vasoconstriction and vasodilation, blood coagulation, fibrinolysis, antigen presentation, atherogenesis and catabolism of lipoproteins. Moreover, it has been recognized that there is considerable heterogeneity in endothelial cells derived from different locations within the body, which is related to the microenvironmental factors acting in each different organ and the specialized function for the endothelium therein (Zetter 1988; McCarthy *et al.* 1991). This structural and functional diversity of endothelial cells has been investigated between different endothelial cell populations including arteries and veins (Lawson *et al.* 2001), large and small vessels (Muller *et al.* 2002) and normal and tumour vessels (Carson-Walter *et al.* 2001), and is the result of molecular differences between endothelial cell populations, which must be taken into account when selecting endothelial cells for *in vitro* analysis. Furthermore, there are species differences which should not be ignored, for example most human endothelial cells bind Ulex europas agglutinin I (UEA-I), whereas murine endothelial cells do not (Gumkowski *et al.* 1987).

Until relatively recently, the most commonly used human endothelial cell for *in vitro* angiogenesis assays was human umbilical vein endothelial cells (HUVEC), which are easily isolated by perfusion of the umbilical vein with trypsin or collagenase and have been successfully cultured since 1973 (Jaffe *et al.* 1973). However, as angiogenesis commonly involves the microvasculature rather than the macrovasculature, these are far from ideal as there are differences between the lineage of these, which may lead to inconclusive or inaccurate responses.

As isolation techniques have become more sophisticated, further endothelial cell types including microvascular as well as macrovascular endothelial cells have been isolated from different organs. Use of endothelial cells from the appropriate organ was shown to be important over 20 years ago following some elegant experiments, which demonstrated glioma cells showing preferential adhesion to brain derived endothelial cells and teratocarcinoma cells adhering most avidly to ovary derived endothelial cells. In contrast, the two tumour types showed no differential adhesion when plated onto 'neutral' fibroblasts (Alby & Auerbach 1984). For

many years, isolation of human endothelial cells from different organs was considered difficult, both in terms of gaining ethical approval and access to tissue, and in terms of the protocols. However, a number of commercial companies have now recognized the requirement for human endothelial cells from different organs and over 19 different types of endothelial cells are now available as primary cell cultures, including pulmonary, uterine, cardiac, bladder, dermal and lymphatic microvascular cells (Figure 1).

A majority of endothelial cells within the body are naturally quiescent, whereas cultured endothelial cells adapt to proliferate *in vitro*, which may be a potential problem when assessing pro-angiogenic factors, as these cells are already exhibiting an angiogenic phenotype. Moreover, whilst proliferating in culture the endothelial cells start to lose their organ specific phenotype and undergo changes in activation state, karyotype, expression of cell-surface antigens and growth properties (Jackson & Nguyen 1997). Even when using primary cultures, it should be taken into consideration that the cells can only be passaged a few times before they lose their 'normal' physiological properties. For example, blood–brain barrier endothelial cells that specifically express P-glycoprotein lose this expression almost immediately *in vitro* (Aird 2003).

Despite the fact that endothelial cells are naturally quiescent *in vivo*, researchers have attempted to immortalize endothelial cells for use in angiogenesis assays. Several methods have been used to immortalize both microvascular and macrovascular endothelial cells including stable transfection of human dermal microvascular endothelial cells with the simian virus SV40 large antigen (HMEC-1 cells; Ades *et al.* 1992), or engineering human telomerase catalytic protein (hTERT) into primary microvascular endothelial cells (HMVEC; Shao & Guo 2004). However, there are inherent problems with this approach, not least that altering the original cells to proliferate in culture will change their angiogenic potential, but also gene expression is likely to be altered and cells may still undergo phenotypic changes with repeated passaging. Indeed when HMEC-1 cells were originally generated, they formed tubules *in vitro* (Ades *et al.* 1992), whereas 12 years later, this is no longer the case (Nisato *et al.* 2004).

Endothelial cells are also likely to behave differently when in tubular structures, in contact with accessory cells such as pericytes and smooth muscle cells, and in response to flow and shear stress. Thus any assays using endothelial cells alone, while likely to be reproducible, readily quantifiable and rapid, will not permit the study of the more complex

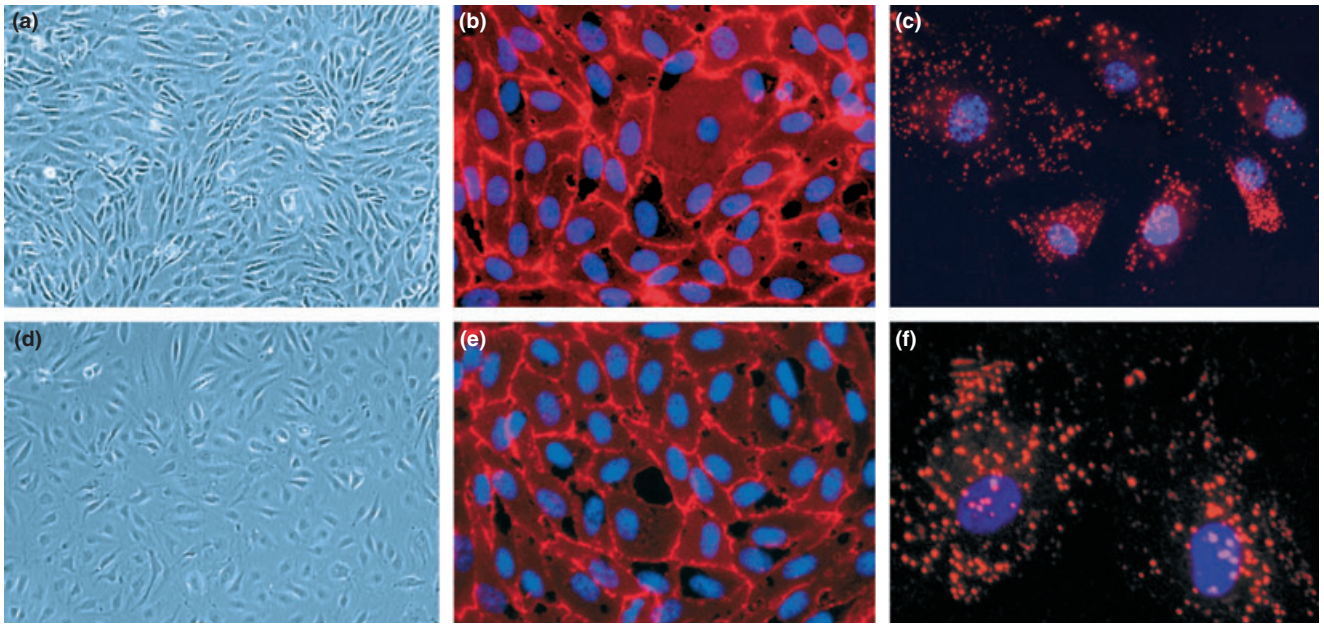

Figure 1 Endothelial cells. Representative photographs of confluent human umbilical vein endothelial cells (HUVEC; a–c) and human dermal microvascular endothelial cells (HuDMEC; d–f) as seen with phase contrast microscopy (a, d), or stained with CD31 (b, e) or von Willebrand's Factor (c, f). In the fluorescent photographs, the nuclei are counterstained with DAPI. The morphology of both cell types is very similar although the HUVEC form a more classical cobblestone pattern. Photographs courtesy of Promocell GmbH, Heidelberg, Germany.

physiological interactions that occur *in vivo*, including the assessment of any indirect effects of an agent e.g. an effect exerted on non-endothelial cells that then produce a factor/molecule, which acts on endothelial cells. This does not mean that assays involving endothelial cells alone are not important, rather that care should be taken with interpretation, and that multiple *in vitro* assays should be used and followed up with one or more *in vivo* assay.

Endothelial cell proliferation assays

Within the process of sprouting angiogenesis, endothelial cells undergo proliferation and as proliferation assays are highly reproducible, easy to perform and generate precise quantifiable data, they are commonly used. However, although endothelial cells in culture are capable of cell division, they eventually become senescent and so proliferation studies need to be carried out soon after isolation; commonly cells are used at passages 3–6 in our laboratory. Appropriate cell density is very important for proliferation studies as a marked reduction in proliferative activity occurs when the cells reach confluence because of cell–cell contact inhibition, diminished nutrient supply and accumulation of cell-derived waste products. Therefore, the confluence of endothelial cells must be less than 70% for evaluating cell proliferation. Moreover, as endothelial cells *in vivo* are usually quiescent, in order to detect the activity of a pro-angiogenic substance it is often necessary to induce a quiescent state in the endothelial cells prior to the experiments. This is usually achieved by serum starving the cells (reducing serum levels to 1%) and then re-introducing full serum and/or growth factors/test substances for the course of the experiment. Care must be taken not to stimulate apoptosis because of starvation.

Cell proliferation can be described as the number of cells dividing, and the easiest way of measuring this parameter is by determining the net cell number. In these assays, a defined number of endothelial cells are seeded and after a certain period of time to allow proliferation to occur (as the cell cycle for endothelial cells is between 18 and 24 h, the time usually chosen is in multipliers of 24 h) the increase in the cell number is measured by a cell counting device such as a haemocytometer (using a light microscope), a coulter counter (an electronic particle counter) or a Vi-cell counter (which measures cell viability as well as number). The haemocytometer is time consuming, requires a high density of cells and is more prone to sampling error, however, it does allow a visual estimation of cell death which, when combined with trypan blue, can be used to estimate cell viability. While the coulter counter is more convenient, accurate, rapid and can be used to count low density of cells, it is more expensive and results might not always reflect cell proliferation, for instance a decrease in cell number may be as a result of cell death rather than a decrease in proliferation. The more modern Vi-cell incorporates trypan blue exclusion to give total number of cells as well as the percentage of dead cells making this the most accurate, but also the most expensive method.

Another method in common use is MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is a yellow tetrazolium salt taken up by cells and cleaved by a mitochondrial dehydrogenase enzyme to produce purple/blue formazan crystals, which accumulate in living cells. These crystals are dissolved by the addition of DMSO and the solubilized formazan product is quantified using an ELISA plate reader. Correlation between production of the formazan product and cell number has been shown (Denizot & Lang 1986; Wemme *et al.* 1992). However, although this method provides rapid detection of cell proliferation, some chemical reagents may alter the activity of the dehydrogenase thereby affecting formazan production without a concurrent change in cell number, and different cell lines may have different absorbance levels even at similar degrees of cell confluence.

DNA synthesis, an alternative measure of cell proliferation, is assayed by measuring the incorporation of [^{3}H]Thymidine into the DNA of the cell using scintillation counting where the amount of radioactivity is proportional to the neosynthesis of DNA (Yu *et al.* 2004). More recently, the use of bromodeoxyuridine (BrdU), which competes with thymidine for incorporation into the DNA during the S-phase of the cell cycle, has replaced the use of radiolabelled thymidine as the incorporated BrdU can be detected by immunocytochemistry at an individual cell level or by ELISA for the cell population. Similarly, it is possible to stain the cells with a DNA binding dye and assess the amount of bound dye using a colourimeter or ELISA reader. However, with all these methods, an increase in labelling may actually be because of DNA repair activities not involved in cell mitosis and a decrease may be because of cytotoxic rather than cytostatic effects of a test substance. An alternative method is to measure the expression of PCNA (proliferating cell nuclear antigen) in cells. PCNA expression increases during G_1-phase, peaks during the S-phase and decreases during G_2/M-phases of the cell cycle and so can be used to distinguish proliferating cells from resting cells. Furthermore, immunocytochemical analysis examining the expression of PCNA in the cell may be employed to provide visual and quantitative evidence of cell proliferation. However, PCNA is also involved in DNA repair and as such may be

expressed by cells that are not proliferating. Thus, incorporation of thymidine analogues or expression of PCNA does not always correlate with cell proliferation (Neckers *et al.* 1995).

Direct cell-cycle analysis using DNA-binding molecules with flow cytometric analysis is an alternative method for analysing proliferation. Again, this method commonly uses BrdU incorporation into cellular DNA followed by saturation staining with propidium iodide (PI), which provides a measure of the total DNA per cell. A correlation between the BrdU content of the cells with that of PI measured using the fluorescence activated cell sorter (FACs) generates the standard cell cycle information (Gomez & Reich 2003). This has the advantage of demonstrating the cell-cycle distribution, proliferative state and a measure of apoptosis within a population, as well as being able to analyse a large number of cells in a short space of time. However, disadvantages include the potential that cellular autofluorescence may cause difficulty in analysis and that large numbers of cells are required.

In order to achieve the most reliable results with proliferation assays, the combined use of two or more methods is strongly recommended; usually some form of cell counting (MTT or direct numbers) combined with a measure of DNA synthesis and cell death analysis.

Endothelial cell migration assays

During the process of angiogenesis, endothelial cells are stimulated to degrade the basement membrane and migrate into the perivascular stroma in response to a gradient of angiogenesis inducing factors including VEGF. Transfilter assays, a modification of the Boyden chamber (Boyden 1962) are the most frequently used assays to quantitatively assess endothelial cell migration. These three dimensional assays are based on the migration of endothelial cells, plated on top of a filter containing 8 μm diameter pores, which allow only active passage of the cells, towards an attractant (test angiogenic factor) placed in the lower chamber (Alessandri *et al.* 1983). The filter may be coated with single ECM proteins such as collagen or fibronectin, or complex matrices, such as Matrigel (Albini *et al.* 2004) in an effort to simulate the *in vivo* microenvironment. This assay has several advantages including high sensitivity to small differences in concentration gradients (Falk *et al.* 1980), high reproducibility, short duration (4–6 h) meaning that there are no proliferative effects and/or matrix synthesis. Theoretically, should the agents alter the location or expression of endothelial cell specific proteins, it would also be possible to examine the filter for those specific changes. Importantly, this assay can be used to distinguish between chemotaxis (directional migration in response to a gradient of soluble stimuli) and chemokinesis (random motility), by generating positive, negative and null gradients by altering the concentrations of reagents in the upper and/or lower chambers (Taraboletti *et al.* 1990). However, disadvantages with this assay are the technical difficulties involved in setting up the assay, maintaining transfilter gradients for prolonged periods of time (as this equilibrates between chambers over time), a lack of specific information about the gradient generated, the inability to observe cell motion during experiments, and the fact that cells migrate through a 'foreign' matrix (polycarbonate or polypropylene filter) (Cary & Guan 1999; Smith *et al.* 2004). Counting cells by eye is time consuming, and it is difficult to obtain accurate and statistically significant results with small numbers of cells crossing the filter, especially when the distribution and/or staining of the cells are/is uneven. The use of computers equipped with digital cameras capable of scanning the filter systematically and software that recognizes stained cells have been used to complement counting by eye. However, the software cannot always distinguish between the pores of the filter and the stained cells (Debeir *et al.* 2004). An alternative indirect method of measuring migration in the modified Boyden chamber involves staining migrating cells with crystal violet, eluting the stain and transferring to a microplate for spectrophotometric analysis whereby the density of the stain correlates with cell migration (Santiago & Erickson 2002).

An adaptation of the Boyden chamber, which replaces the transparent filter with a light shielding material such as polyethylene terephthalate, enables a time course endothelial cell migration to be established by fluorescently labelling the endothelial cells. In this manner, non-migrating endothelial cells above the filter are invisible to the fluorescent plate reader and only the migrating cells are counted (Goukassian *et al.* 2001).

A further modification of the Boyden chamber enables the study of pseudopodia extension, an event involved in cell migration (Parent & Devreotes 1999). By altering the pore size of the filter to 3 μm and coating the membrane on both sides with an ECM protein, the cells will extend pseudopodia through the pores in the direction of the gradient, and altering the gradient will alter the number of pseudopodia extended through the membrane. Removal of the cell body from the upper surface enables determination of the total pseudopodia protein on the lower surface of the filter (Klemke *et al.* 1998).

An alternative type of migration assay is based on the premise that endothelial cell migration into a denuded area is a pivotal event in wound healing *in vivo*. A scraping tool is

used to remove a portion of a confluent endothelial cell monolayer to provide a margin from which the endothelial cells migrate to fill the denuded area (Figure 2; Wong & Gotlieb 1984). The rate and extent of endothelial cell migration is then monitored microscopically at various time-points or by time-lapse microscopy (Pepper *et al.* 1990). This two dimensional *in vitro* assay is considered to represent one aspect of wound healing in as much as the confluent endothelial monolayer has been 'wounded' by scraping and the cells are migrating back to re-form the monolayer (Lampugnani 1999). The major advantage of this method is that cell migration can be monitored over time, thus estimating a rate of migratory response. However, fluorescence and immunofluorescence microscopy combined with time-lapse cinematography used to correlate the cytoskeletal events with motile activity of cells occurring during the 'healing of the wound' have shown that 'wound repair' in this model is a multi-step process involving spreading, proliferation and migration events (Wong & Gotlieb 1988; Coomber & Gotlieb 1990). This means that this assay is inaccurate for the determination of the net migration effect. Moreover, quantification is difficult and arbitrary, results may vary depending on size of wound and cell growth, results may be compromised by release of substances from damaged endothelial cells, and it is technically difficult to ensure that control and experimental groups are run under identical conditions of confluence and that the area of denudation is precise (Auerbach *et al.* 1991; Cai *et al.* 2000).

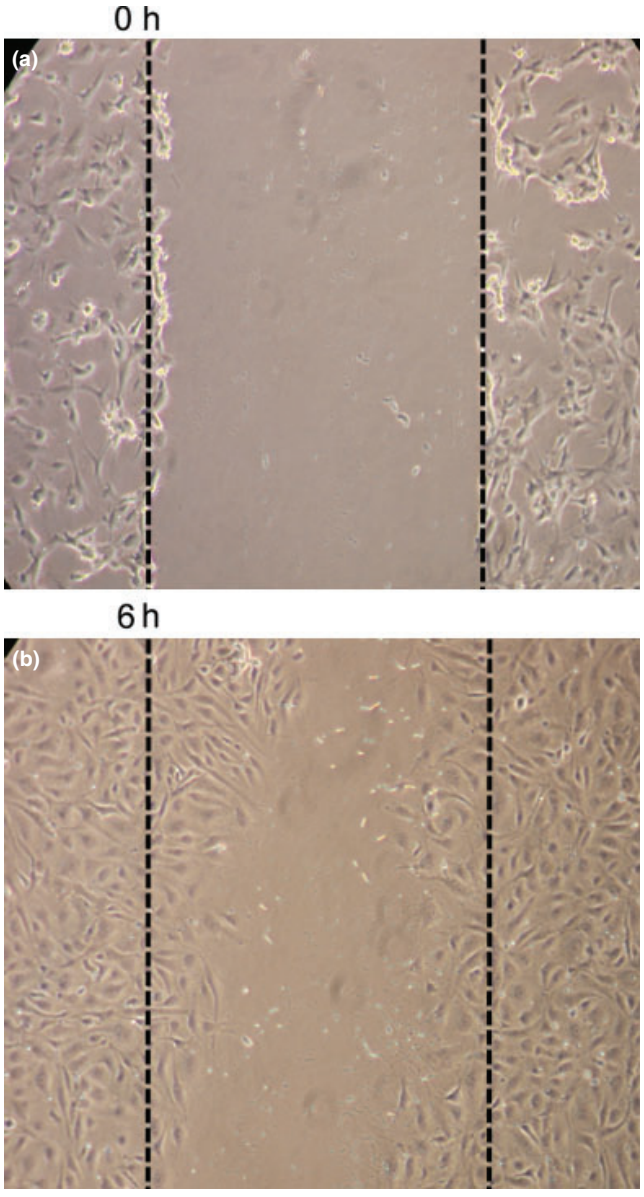

Figure 2 'Wound healing' migration assay. The pictures show HuDMEC cells that have been (a) freshly wounded and (b) six hours after wounding at which point cell migration into the area of the wound can clearly be seen. Dotted lines mark the edge of the 'wound'. Photographs courtesy of Chryso Kanthou, University of Sheffield, UK.

An attempt to remove the problems of generating identical areas of denudation and damaged endothelial cells resulted in the Teflon fence assay whereby endothelial cells are confined to a region of a well with a Teflon fence and allowed to proliferate until confluency. The fence is then removed and over time, the cells will migrate into the new area available. Cells are fixed and then counted at specific times following fence removal and at specific distances from the site of the initial population (Cai *et al.* 2000). Using this method, it is possible to assess the migration of endothelial cells on gradients of surface bound ligands (Smith *et al.* 2004). However, this is technically difficult to set up as producing increasing gradients is time-consuming and laborious, and net migration is difficult to discriminate from proliferation when experiments exceed 24 h.

An increase in overall endothelial cell motility can also be a measure of an angiogenic response and phagokinetic track methods can be used to assess this. The first version of this assay used colloidal gold-plated coverslips as a substrate for the movement of cells, which displace the colloidal gold leaving a track that can be measured for directional properties and total distance (Zetter 1987). More recently, the assay was modified to permit rapid preparation and screening of 96-well plates. A monolayer of 1 μm polystyrene beads is deposited on the bottom of the plate and endothelial cells settle on the bead monolayer and generate tracks similar to those produced in colloidal gold (Obeso & Auerbach 1984). The major advantage of this method is that

accurate measurement of cell motility can be achieved, and that it is possible to study directional effects on cell movement. This assay has proved equally useful for studying anti-angiogenic agents such as endostatin (Auerbach *et al.* 2003) and pro-angiogenic factors such as VEGF (Albrecht-Buehler 1977). However, its major disadvantages are that the cells are migrating on an unnatural/non-host substrate (not one found *in vivo*), only a small number of cells can be tracked, and the assays are time consuming to analyse and difficult to interpret, and requiring the use of complicated computational analysis software to track the cell paths recorded using time-lapse microscopy (Ariano *et al.* 2005).

The choice of migration assay depends upon the compound being tested (i.e. is it soluble or surface bound) and the questions being asked. While the Boyden chamber and adaptations thereof, have the advantages of allowing both chemotaxis and chemokinesis to be quantified, the relatively simple scratch assay may be more appropriate when investigating wound healing as the cells at the edge of the scratch are likely to be damaged by the process. However, for the direct measurement of cell motility and directional effects on cell movement, the phagokinetic track method is the most appropriate.

Endothelial cell differentiation assays

Assays that simulate the formation of capillary-like tubules are regarded as representative of the later stages of angiogenesis (differentiation) and are used extensively to assay novel compounds for pro- or anti-angiogenic effects. The basic tubule formation assay involved plating endothelial cells onto or into a layer of gel matrix (commonly collagen, fibrin or Matrigel), which stimulates the attachment, migration and differentiation of endothelial cells into tubule like structures in a manner that simulates the *in vivo* situation (Lawley & Kubota 1989; Kanzawa *et al.* 1993). These assays are usually run in the presence of potential modulators of angiogenesis and tubule development is observed over a 4- to 24-h period and recorded using a digital camera. The formation of tight junctions between the endothelial cells in the developing tubules has been confirmed by electron microscopy (Auerbach *et al.* 2003), although these early structures lack lumen.

The type of matrix used is important as different matrices result in different rates of differentiation, for example, culturing endothelial cells on collagens I and III (interstitial collagens) results in cell proliferation with only occasional tubule formation, whilst plating on collagens IV and V (basement membrane collagens) leads to extensive tubule formation with only minimal proliferation (Madri & Williams 1983). Matrigel, a mixture of extracellular and basement membrane proteins derived from the mouse Engelbreth-Holm-Swarm sarcoma, was found to be the most potent matrix for tubule formation, with tubules beginning to form within 1 h and completely formed within 12 h following endothelial cell plating (Lawley & Kubota 1989). More recently, a growth factor reduced Matrigel, in which the levels of cytokines and growth factors have been markedly reduced, has become available and avoids problems associated with the over-stimulation of endothelial cells that occurs in standard Matrigel (Figure 3a). However, although Matrigel has been shown to direct endothelial cells along the differentiation pathway (Fawcett 1994), there is some debate as to whether tubules generated on Matrigel represent capillaries, as the presence of lumen has been shown by some groups using electron and light microscopy (Grant *et al.* 1991; Connolly *et al.* 2002) and disputed by others who could not observe lumen (Bikfalvi *et al.* 1991). Interestingly, it appears that the mechanisms involved in the differentiation of endothelial cells to form tubules are at least partly dependent upon the matrix the cells are plated onto (Zimrin *et al.* 1995; Cockerill *et al.* 1998; Segura *et al.* 2002); therefore, to confirm the action of any test substance, the assays should be performed on more than one matrix. It should also be noted that other non-endothelial cell types including

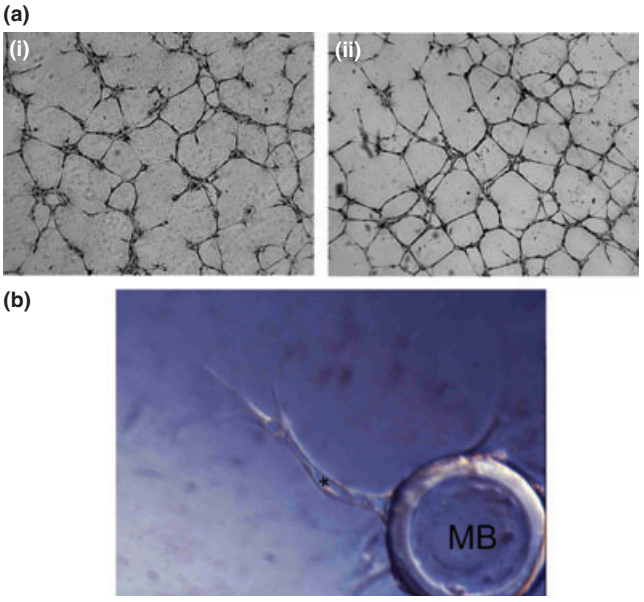

Figure 3 Tubule formation assays. (a) Appearance of HuDMEC forming tubules when plated onto GF-reduced Matrigel in the absence (i) or presence (ii) of VEGF (10 ng/ml). (b) Appearance of tubules complete with lumen (star) formed by endothelial cells on microcarrier beads in a fibrin gel.

primary human fibroblasts, human prostate carcinoma cell line (PC3) and glioblastoma cells (U87-MG) have been shown to form 'tubules' on Matrigel (Donovan *et al.* 2001) and therefore the results should interpreted with caution. Moreover, care must be taken in setting up the assays using the optimum amount of matrix to ensure an even coating on the well, and the correct density of endothelial cells, both of which may affect the tubule response to growth factors (Liu *et al.* 2002).

Four primary variables are commonly used to determine the extent of tubule formation, namely average tubule length, number of tubules, tubule area and number of branch points. Each of these variables can be measured manually or by using image analysis programs. However, care must be taken to ensure that the tubules are distinguished from the undifferentiated proliferating islands of cells and the background when using image analysis software. Moreover, it is possible that tubule area can actually decrease under angiogenic conditions when cells are plated at high density (Liu *et al.* 2002). Interestingly, different factors will affect different parameters of tubule formation and therefore all four parameters should be measured from a single experiment.

This method has been adapted for 384 and 1536 well formats using complicated computational analysis (Sanz *et al.* 2002), which eliminates some of the problems with respect to reproducibility and difficulties in analysing the whole well. However, the majority of laboratories use a 96-well format and a semi-computational analysis as this is more affordable. The lack of consistent lumen formation and the homogeneous nature of the tubules are major limitations of this assay.

It is also possible to use Matrigel, collagen and fibrin clots to generate 3D tubule formation assays in an attempt to provide an *in vitro* model of tubulogenesis that more closely mimics the *in vivo* situation. The most commonly used of these assays sandwiches endothelial cells between layers of matrix (Matrigel, collagen or fibrin). For the first 7 days of the assay, the cells form tubules in the horizontal plane, but by day 15 the endothelial tubules branch upwards and penetrate into the gel to form a 3D network of tubules (Gagnon *et al.* 2002). An alternative is to coat microcarrier beads with endothelial cells and then to disperse the beads throughout the gel (fibrin clots are most commonly used for this) and in 7 days, tubules will form (Sun *et al.* 2004) (Figure 3b). However, one limitation of this assay is that the microcarriers can sink to the bottom of the gel. A further limitation common to all the 3D assays is that the gel has to be relatively thin to allow the diffusion of oxygen and nutrients; otherwise excessive cell death occurs. While these assays do most closely mimic the *in vivo* situation, analysis of the tubule formation in three dimensions remains a challenge. The analysis involves taking images at different heights within the gel and the length (width and height) and the largest diameter of each vessel are measured (Gagnon *et al.* 2002). Alternatively, gels can be fixed, dehydrated, sliced into 5-μm sections and stained with haematoxylin and eosin (Ilan *et al.* 1998). The microvessel density can then be assessed in each slice, or at different heights through the gel by using an eyepiece mounted 25-point Chalkley array graticule and counting the number of points on the Chalkley grid coinciding with tubules (adapted from Fox *et al.* 1995). The limitations are obvious with only a proportion of tubules, area or volume of the gel being analysed. Moreover, these assays take considerably longer to run (5–15 days).

Another form of tubule formation assay involves the co-culture of endothelial cells with stromal cells, with or without the provision of an ECM (Montesano *et al.* 1993; Nehls & Drenckhahn 1995; Bishop *et al.* 1999). The stromal cells used can be fibroblasts, smooth muscle cells or blood vessel explants (see later) containing numerous cell types (Nicosia & Ottinetti 1990; Sakuda *et al.* 1992). When human fibroblasts are co-cultured with endothelial cells, the fibroblast secretes matrix components that act as a scaffold and enables the endothelial cells to form tubules that contain lumen, with more heterogeneous pattern of tubule lengths, which more closely resemble the capillary bed *in vivo* (Bishop *et al.* 1999; Donovan *et al.* 2001). This assay is time consuming (12–14 days) and, as the matrix components secreted by the fibroblasts have not been defined, this assay is less well-characterized. Analysis is usually limited to fixing and staining the endothelial cells and then carrying out analysis of tubule length, number of tubules and branch points. An alternative method of analysing tubule formation in this assay is to use an alkaline phosphatase-coupled anti-CD31 antibody linked to a soluble chromogenic substrate and then using an ELISA (Friis *et al.* 2003); however, this will only measure changes in cell number, not differentiation *per se*. An alternative co-culture differentiation assay is the collagen-bilayer system, which has a layer of collagen containing fibroblasts or epithelial cells followed by a layer of cell-free collagen and then finally endothelial cells are seeded on top. Tubule development occurs in 15–20 days, is in three dimensions and is recorded using a phase contrast microscope and a camera (Montesano *et al.* 1993). Quantification of this assay is difficult because of the three dimensional nature and there are problems with gel thickness leading to the death of the fibroblasts or epithelial cells.

There is no doubt that there is a great deal of choice when it comes to differentiation assays. The main differences

between the 2D differentiation assays are the choice of matrix on which the endothelial cells differentiate (collagen, fibronectin, fibrin or Matrigel), and the choice of the most appropriate matrix will depend upon the type of angiogenesis being studied, e.g. fibrin would be the most appropriate for wound healing where endothelial cells migrate into a fibrin clot as part of the healing process. While a 3D assay is likely to be more representative of angiogenesis *in vivo* than the 2D assays, there are technical challenges in both setting up the assays and in fully analysing them. The co-culture assays have the advantage of including supporting cells (usually fibroblasts), which secrete a matrix for the endothelial cells to differentiate upon; however, as this assay lasts up to 2 weeks, the results measure proliferation as well as differentiation. The limitation of all the assays is the time consuming nature of the analysis, which means that although the most reliable results are likely to be obtained by using at least one 2D and one 3D assay, in reality the majority of groups use a single 2D differentiation assay.

Endothelial–Mural cell co-culture assays

After endothelial cells have coalesced to form tubules, mural cells are recruited to maintain vessel quiescence and stability, and therefore an essential part of the study of angiogenesis is the interaction between endothelial and mural cells. The simplest co-cultures involve seeding the cells simultaneously or allowing one cell to adhere type first and then seeding the second cell type on top. In order to assess the influence of one cell type on the other's behaviour, it is necessary to label at least one population prior to seeding (Hirschi *et al.* 1998). Fluorescently labelled cells are especially useful as they enable the monitoring of cell behaviour (migration, proliferation, tubule formation) in real time over the course of the experiment. In order to assess the effect of one cell type on the proliferation of the other, the cells can be labelled *in situ* in the co-culture well with BrdU and then fixed and immunostained with antibodies against BrdU to identify proliferating cells in combination with co-staining with cell type specific markers to enable the growth of each population to be quantified (Hirschi *et al.* 1999). Such assays have been used to study the effects of endothelial cells on mesenchymal cell differentiation (Hirschi *et al.* 2001) and the analysis of gap junctions (Hirschi *et al.* 2003) using a dual whole-cell voltage clamp to determine to what extent fluorescently labelled endothelial cells communicate specifically via gap junction channels and the impact on endothelial-induced mural cell differentiation. The addition of cells from a genetically altered background can greatly enhance the usefulness of this assay as a tool for identifying any significant interactions between endothelial and mural cells in angiogenesis. This method is simple to set up and analyse using simple labelling techniques; however, it is not possible to assess the extent of cell–cell interactions mediated via diffusible paracrine factors released by one cell type or directed migration of cells, as the two cell types are dispersed evenly through the culture dish.

Alternatively non-contact co-culture assays can be used such as the 'under agarose assay' where agarose is set in a well, two holes are punched and the different cell types seeded into each hole where they attach to the bottom of the well. Over time, the cells migrate towards each other under the agarose (Hirschi & D'Amore 1998). The alternative to this is the transwell where one cell population is plated on the bottom of the well and the other population is plated on the insert so that the second cell type are suspended above the first. Both co-culture types allow for the exchange of soluble diffusible signals that can modulate growth and migration, which can both be measured using methods described previously. Specific neutralizing reagents can be added to determine whether the cellular effects are suppressed. The under agarose assay has the advantage that if the cells are left in culture for 5–7 days, the cell types can come into contact with one another and the effects of cell–cell interactions on cell phenotype can be monitored (Hirschi *et al.* 1998).

Like the tubule formation assays, endothelial–mural cell co-culture assays in three dimensions have been developed. The first method used was a spheroid method where endothelial cells and mural cells are seeded together in non-adherent round bottom 96-well plate and spheroids will form between 1 and 4 days (Korff *et al.* 2001). However, unlike the *in vivo* situation, the mural cells are in the middle and the endothelial cells have not formed tubules. The alternative method is similar to the 3D tubule assays where endothelial and mural cells are sandwiched between layers of Matrigel and tubules invade into the Matrigel (Darland *et al.* 2003). This model more closely resembles the *in vivo* phenotype where mural cells are recruited by endothelial cells to form a supporting mural cell layer. However, there are technical challenges associated with these assays, and conditions of flow, which also alter endothelial cell phenotype (Akimoto *et al.* 2000), have not been included.

While the use of mural–endothelial co-cultures are undoubtedly advantageous in revealing information regarding the interaction between the cell types, it is important to consider the question being asked before choosing which of these assays to use. Direct contacting assays will enable the evaluation of the direct effects of one cell type on another, whilst the non-contacting assays enable measurement of

substances released by the cells. Although the assay most representative of the *in vivo* situation is undoubtedly the 3D Matrigel sandwich tubule assay, this is difficult to analyse fully, and does not reveal information about soluble diffusible signals released from each cell type.

Ex vivo *assays (organ culture)*

The recognition that angiogenesis *in vivo* involves a network of endothelial cells and their surrounding cells in constant communication with the microenvironment has led to the development of organ culture methods in an attempt to evaluate this complex process. These *ex vivo* assays are all similar, in that segments, discs or sections of the specific tissue type are cultured in a three dimensional matrix *in vitro* and are monitored for microvessel outgrowths over a period of 10–14 days. Quantification in these assays is challenging and needs to be standardized to allow interpretation of results. In general, quantification is achieved by measuring the number and length of microvessel outgrowths from the primary explants (Nicosia *et al.* 1997), although in practice as the outgrowths are often clustered together, the area covered by outgrowth is more commonly measured. Accurate quantification requires determining the number and length of branching microvessels, the size and form of the original tissues, and the number and spatial distribution of fibroblast-like cells (Blacher *et al.* 2001). These are counted by eye (number of vessels and branch points; Brown *et al.* 1996) or digitally (area, sprouting index (ratio between area of outgrowth and area of original tissue), or the mean area density; Kruger *et al.* 2000), however, length and width of the microvessels should also be taken into account (Go & Owen 2003). A thin preparation modification of the assay allows for the staining of aortic outgrowths as whole mounts thereby easing quantification (Zhu & Nicosia 2002).

These explant assays are considered by many to essentially simulate the *in vivo* angiogenesis environment because the system includes the surrounding non-endothelial cells (such as smooth muscle cells and pericytes) and a supporting matrix. In addition, the endothelial cells have not been altered by repeat passaging and are thus quiescent at the time of explantation making the system more representative of the *in vivo* situation where angiogenesis is stimulated and quiescent endothelial cells respond by proliferation, migration from the existing vessels and differentiating into tubules (Staton *et al.* 2004a). However, the model is not entirely representative of the microvascular environment encountered during disease states, such as tumour growth, as the large number of different factors released by tumours and the tumour cells themselves are not present (Auerbach *et al.* 2000). Nonetheless, this assay has the advantage of low cost, easy manipulation of treatment conditions, lack of inflammatory complications seen with *in vivo* models, and the possibility of generating many assays from one animal as approximately 30 rings may be obtained from each isolated rat aorta (Kruger *et al.* 2001).

Although the aortic ring assay is the most commonly used, there are reported problems with variability of results between explants from different animals, which is why the adaptation to generate 30 rings from one animal is so valuable (Kruger *et al.* 2001). The porcine carotid artery explant assay is considered an improvement on the rat aortic ring assay, as the larger pig vessel enables greater numbers of assays to be performed from one animal thus eliminating statistical variation between animals (Stiffey-Wilusz *et al.* 2001). However, this has the obvious drawback that the single donor may not be truly representative of the population. A modification of this assay with aortic fragments from mice has been used to show differences in aortic disc angiogenesis in tumour bearing *vs.* non-tumour bearing mice, as well as differences when mice are treated with an oral anti-angiogenic agent (Berger *et al.* 2004). Moreover, the mouse aortic assay can be used to study differences in gene function in transgenic or knockout mice (Devy *et al.* 2002). However, angiogenesis is a microvascular process, and therefore the use of large vessels in these assays is far from ideal.

A further modification of the rat aortic ring assay is the chick aortic arch model (Muthukkaruppan *et al.* 2000), which has the advantage of being a rapid experiment that can be carried out in serum-free conditions. Embryonic arch endothelial cells share many properties with microvascular endothelial cells, however, these aortic arches are derived from growing embryos, and hence will be proliferating prior to explantation and are therefore not truly representative of the stimulation of non-proliferative endothelial cells that occurs *in vivo*. An alternative assay is the use of 17-day-old foetal mouse metatarsals, which contain microvascular endothelial cells in the perichondrium and when cultured in medium containing serum, the dissected metatarsals show vessel outgrowth after 10 days of culture, which stain positively for endothelial cell markers (Deckers *et al.* 2001). However, like the chick aortic arch assay, these metatarsals are derived from growing embryos, which undergo rapid cell division before explantation and exposure to angiogenic mediators.

The most recent development of an *ex vivo* model has focused on angiogenesis and the relevance of using vena cava explants, as angiogenesis typically originates from the venous side of the vascular bed (Folkman 1982). A vena cava–aorta model has therefore been developed with rings cut from both the aorta and vena cava of an animal

explanted in the same culture dish. This adds an additional layer of complexity while closely mimicking the environment *in vivo* where both venous and arterial vessels are present. The main challenges are the technical difficulty in acquiring the vena cava tissue, and the fact the venous explants have a tendency to collapse (Nicosia *et al.* 2005). It remains to be seen whether this co-culture system is a more sensitive assay and better screening tool for evaluating potential anti-angiogenic agents.

A major problem with all the assays described so far is the use of non-human tissues, which questions their applicability as preclinical screening assays, because the responses seen with various drugs/test substances may be species specific. In an effort to overcome this species-specificity issue, human explant models have been developed using human placental or umbilical blood vessels and saphenous veins (Brown *et al.* 1996; Jung *et al.* 2001; Kruger *et al.* 2000; Figure 4). However, as the outgrowth of endothelial cells in these explants is derived from large vessels, whether this type of assay is truly representative of *in vivo* angiogenesis remains an issue.

In vivo *assays*

The wide range of *in vitro* and *ex vivo* assays discussed in the previous sections are in common use for investigating angiogenesis, however, care must be taken with interpretation and comparisons between different assays published by different groups are often difficult because of differences in the origin of endothelial cells, passage number, substrates and media composition used. Interestingly a substrate, which has efficacy *in vitro*, may not show any activity *in vivo* (Liekens *et al.* 2001) and *vice versa*, with some compounds showing little efficacy *in vitro* having strong activity *in vivo*. Therefore, the *in vivo* evaluation of agents is a vital step in drug development and the current *in vivo* assays are described in the following sections.

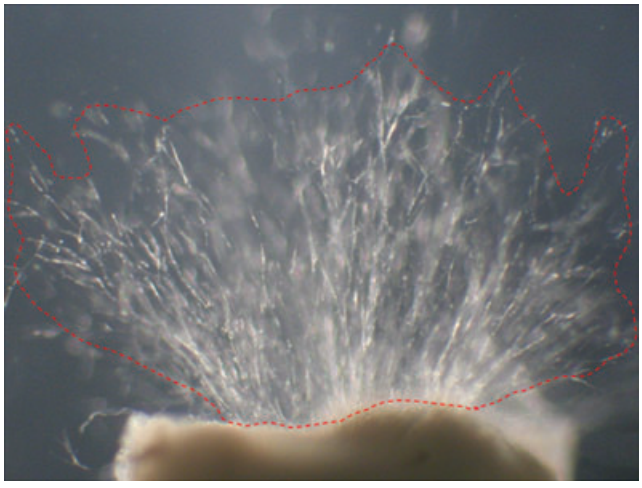

Figure 4 Formation of tubules from a human umbilical artery. The umbilical artery is cultured in full growth medium and the appearance of tubules sprouting out from the artery can clearly be seen (ringed by a dotted line).

Chick chorioallantoic membrane assay

The chorioallantoic membrane (CAM) of the chick presents an accessible system in which to study angiogenesis *in vivo*. As the chick is a living system, although it is simple, this model provides a more physiological system for *in vivo* analysis of cells, pathogens and pharmacological reagents than the *in vitro* systems previously described. The CAM is readily accessible outside the embryo and provides a technically simple way of studying complex biological systems. The chick is relatively immunotolerant, which enables the study of cross-species xenografts including tumours (Vogel & Berry 1975), cultured human cancer cells (Auerbach *et al.* 1975) and mammalian tissue explants (Ausprunk & Folkman 1976) for extended periods of time. Alternatively, a test substance can be placed on the CAM, through a window cut carefully in the eggshell, in either slow release polymer pellets, in gelatin sponges or air-dried onto filter discs. There are two main methods of accessing the CAM, one is by allowing the embryo to develop in the shell and then cutting a window in the shell, and the other is to culture the embryo in a Petri dish, without the shell (Zijlstra *et al.* 2006). Both techniques are minimally invasive, and while the 'in shell' technique requires less maintenance and embryos can be maintained to later stages of development, the '*ex ovo*' method provides improved access to the test site, improving the ability to repetitively treat or to have multiple test sites on one CAM, and image a wider area of the CAM. Angiogenesis is typically allowed to proceed for 3 days, although longer time periods have been used. The measurement of angiogenesis occurs by removal of the area of CAM around the filter disc or xenograft and then use of a dissecting microscope to count the number of vessels, number of branch points or alternatively to score the vascularity on a semi-quantitative scale of 0–4.

There are many advantages to the CAM assay including the fact that it is relatively simple and inexpensive and therefore suitable for large-scale screening. The CAM is also amenable to biochemical analysis and extracts of CAM have been used to demonstrate signalling events *in vivo* (Alavi *et al.* 2003). Furthermore, it is possible to apply test

substances directly or via intravenous or intra-allantoic injections, and the lack of excretion permits maintenance of the test reagents in circulation for extended periods. In addition, accessibility of the CAM permits serial application to be easily performed. However, there are also important limitations that must be taken into account. The CAM itself is well vascularized; hence, to distinguish new capillaries from the existing ones can prove difficult. Moreover, as developmental angiogenesis occurs within the CAM up to 11 days, results obtained from embryos younger than this have the additional complication that unspecified interactions with endogenous factors may be affecting angiogenesis. Therefore, in most cases it is preferable to wait until day 11. Furthermore, chemical or physical irritation of the CAM, including shell dust generated whilst cutting the window in the shell, can lead to an inflammatory response, which in itself may induce angiogenesis, thereby hampering identification of the specific responses to the test substance. It is often necessary to wait 3 days after making the window before adding the test substance to check for an immune response or alternatively add an anti-inflammatory agent to the filter discs prior to their use. However, overt suppression of inflammation may not always be desirable, especially as some angiogenic stimuli work in concert with the inflammatory response (Leibovich *et al.* 1987). Finally, the membrane is sensitive to changes in oxygen tension, making the sealing of the window a vital part of the procedure (Auerbach *et al.* 2000).

Zebrafish

Zebrafish (Danio rerio) is a small tropical freshwater fish that under optimal conditions yield hundreds of embryos per mating. These large numbers and the optical clarity of the developing embryos facilitate the study of development and angiogenesis. The zebrafish share many genes and mechanisms of angiogenesis regulation with mammals, making this organism a useful system in which to analyse the development and function of the vasculature (Rubinstein 2003). The formation of the intersegmental vessels and the subintestinal veins in early embryos is well characterized and easily monitored (Isogai *et al.* 2001) making them suitable for the study of angiogenesis inhibitors. However, there has been some debate as to whether angiogenesis or vasculogenesis is being measured, as these processes are not separated definitively or temporally in the developing embryo. The dorsal aorta and posterior cardinal vein are formed by vasculogenesis (Zhong *et al.* 2001) while the intersegmental vessels are thought to be formed by angiogenesis. However, it is possible that in the formation of the intersegmental vessels, angioblasts may not differentiate into endothelial cells prior to sprouting in which case intersegmental vessels would be formed by type II vasculogenesis (Childs *et al.* 2002). There is less debate over the subintestinal veins, which are commonly accepted to be formed by angiogenesis and are increasingly commonly studied (Zheng *et al.* 2007).

The embryos are inexpensive to generate and easy to maintain long-term, with hundreds of offspring produced each week making it possible to perform large-scale screening studies. It is also possible to undertake specific gene knockdown by antisense morpholino oligonucleotides, which allows rapid assessment of gene function in angiogenesis (Currie & Ingham 1996). Moreover, the experiments are relatively short, requiring only small amounts of drug per experiment and do not require great technical expertise. The method used for the treatment depends on the characteristics of the drug in question as small lipophilic compounds can simply be added to the water and will be absorbed by the fish whereas peptides/proteins have to be injected into the yolksacs of the embryos. As the embryos develop outside the mother and are transparent, direct observation and quantification of blood vessel formation is straightforward using a low-power microscope (Lawson & Weinstein 2002).

In order to visualize the developing vasculature, a number of different techniques have been employed including dye injection, which will highlight patent vessels, although the correct size of the dye must be used as small molecular weight dyes leak through permeable endothelial junctions. Alkaline phosphatase (AP) staining is an accepted method for staining endothelial cells, as these cells express relatively high levels of endogenous AP activity (Childs *et al.* 2002). However, this method is the most useful in early embryos (up to 3 days old) and can only be performed on fixed tissue. Until recently, Confocal microangiography has been one of the most powerful tools for visualization of the zebrafish vasculature (Weinstein *et al.* 1995). Briefly, 0.02-μm fluorescent microspheres are injected into the vasculature of living animals and the entire vasculature can be observed using confocal microscopy (Chico *et al.* 2008). However, early steps in vasculogenesis and growing but not yet lumenized angiogenic blood vessels cannot be observed.

More recently, transgenic zebrafish with green fluorescent protein expressed under the control of promoters specific for the developing vasculature have been generated including Fli-eGFP (Lawson & Weinstein 2002), mTie2-GFP (Motoike *et al.* 2000) and Flk-GFP (Cross *et al.* 2003). Of these different transgenics, the Fli-eGFP has the strongest fluorescence, although there is neural crest expression, which can obscure visualization of vessels in the head (Lawson & Weinstein

2002). The advantage of these fluorescent transgenic fish is that high optical resolution imaging of the vasculature in developing animals can be clearly observed over time. A combination of the Fli-eGFP zebrafish injected with red fluorescent microspheres enables the observation of flow within the newly forming vessels (Chico *et al.* 2008; Figure 5). Alternatively, a transgenic zebrafish where erythrocytes are specifically labelled with dsRED, a red fluorescent marker (GATA1-dsRED) has recently been crossed with Fli-eGFP fish to generate a double transgenic (Fli-eGFP/GATA-dsRED) whereby the blood vessels can be observed as green and the erythrocytes as red enabling confocal movies of blood flow to be generated (Gray *et al.* 2007). Tumour angiogenesis has also been observed in transgenic Fli-eGFP zebrafish by injecting fluorescent tumour cells close to the developing subintestinal veins, into the yolk sac, hindbrain ventricle or circulation (Haldi *et al.* 2006; Stoletov *et al.* 2007). In each case, the tumours grow and stimulate angiogenesis, which can clearly be seen by confocal microscopy. However, the relevance of fish endothelial cell angiogenesis in human tumour xenografts is under debate.

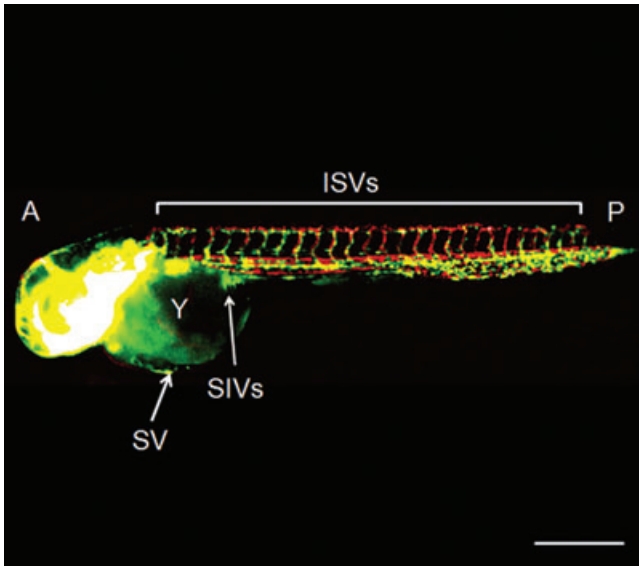

Figure 5 Angiogenesis in the zebrafish embryo. A confocal image of a Fli-eGFP zebrafish embryo showing green blood vessels at 48 h postfertilization. dsRED microspheres were injected into the sinus venosus allowing time lapse confocal laser imaging along with digital motion analysis to track the passage of the dsRED microspheres through the zebrafish circulation. Scale bar, 200 μm. A, anterior; ISV, intersegmental vessels; P, posterior; SIV, subintestinal veins; SV, sinus venosus; Y, yolksac. Image courtesy of Russell Hughes, Philip Ingham & Claire Lewis, University of Sheffield, UK.

Implantation of sponges and polymers

A variety of implanted devices into an animal (commonly mice) have been used for a number of years to study angiogenesis, which allow modulation of the environment in which angiogenesis occurs. The materials utilized have included stainless steel mesh chambers (Schilling *et al.* 1959), hollow chambers with porous walls (Sprugel *et al.* 1987), synthetic sponge matrix (polyvinyl alcohol, polyester and polyurethane) implants or Matrigel plugs (Kleinman *et al.* 1987).

Matrigel is liquid at 4 °C and forms a solid gel at 37 °C. The test substance can be suspended in the gel, which is then injected subcutaneously where it forms a solid plug allowing the slow release of the substance (Passaniti *et al.* 1992). The use of growth factor reduced Matrigel has significantly reduced the problem of the growth factors present within the Matrigel masking the effects of the test substance. A more recent improvement to the assay is the encapsulation of the Matrigel in a plexiglass chamber or within flexible plastic tubing prior to subcutaneous implantation (Kragh *et al.* 2003; Ley *et al.* 2004; Baker *et al.* 2006). The angiogenic response is quantified by (i) sectioning the Matrigel and assessing the extent of the CD31-positive vessel growth into the plug or (ii) measuring the haemoglobin content of the plug. However, neither of these are ideal as sectioning and immunohistochemical staining are time consuming and costly, and haemoglobin content could be increased because of haemorrhage within the plug. An alternative method is the use of FITC-dextran injected into the animals followed by quantification of the FITC-dextran within the implant, which is considered an improvement over the haemoglobin content (Plunkett & Hailey 1990). Matrigel has the advantage that it is a natural substance providing a natural environment in which to initiate an angiogenic response, however, it is costly, analysis is time consuming and because of the presence of growth factors and cytokines (even in the growth factor reduced Matrigel) the extrapolation of results should be made with caution.

A variety of implanted sponges and polymer matrices, which provide an environment of defined dimensions, have been used to induce and study angiogenesis. However, the implants do become encapsulated and elicit the formation of foreign body giant cells (fused macrophages), which secrete angiogenic cytokines, thereby interfering with the test substrate. The intensity and speed of the tissue response elicited by the sponge depend on several factors including the size and type of the sponge material (Salvatore *et al.* 1961). The sponge implant technique has been used to study the formation of fibrovascular tissue measuring neovascularization and

wound healing (Edwards *et al.* 1960), collagen metabolism (Paulini *et al.* 1974), fibronectin deposition (Bollet *et al.* 1958), kinetics of cellular proliferation (Davidson *et al.* 1985), evolution of granulation tissue (Holund *et al.* 1979), the extent of neutrophil and macrophage accumulation into the sponge compartment (Belo *et al.* 2004; Ferreira *et al.* 2004). However, it should be pointed out that some of these will be because of the sponge material itself and not necessarily the test substrate injected into the sponge. These non-specific immune responses can lead to an angiogenic response, so care needs to be taken when interpreting the results. Neovascularization in these models is measured in a variety of ways including immunohistochemical staining (e.g. CD31 or ICAM-2; Solowiej *et al.* 2003; Baker *et al.* 2006), the blood/haemoglobin content of a sponge or the levels of a radioactive tracer in the blood (Plunkett & Hailey 1990). A cannulated version of the sponge model was first described in 1987 (Andrade *et al.* 1987) in which direct blood flow measurement was achieved by a ^{133}Xe clearance technique, and this method has been further modified by using fluorescein in conscious animals to measure the local blood flow. This has been used in combination with tumour cells implanted within the sponge to monitor tumour-induced angiogenesis (Mahadevan *et al.* 1989; Lage & Andrade 2000). The advantage of these techniques is that angiogenesis can be measured in a conscious animal without the need for anaesthesia. However, determination of blood flow is limited to fixed time points and damage of the neovasculature after injection of the tracers often occurs. Other limitations to this method include an inflammatory response, excessive matrix deposition and unwanted fibrosis and a wide variety in responses caused by different materials used (size, structure, porosity, composition, etc.) making results difficult to interpret. Despite these disadvantages, the relative ease of set up, and the ability to incorporate methods to assess blood flow, as well as the possibility of obtaining a large amount of data from this model make it versatile and powerful.

Corneal angiogenesis assay

The cornea is the only avascular transparent tissue in the body, so any vessels penetrating from the limbus into the corneal stroma are newly formed, readily visible and can be quantified (Gimbrone *et al.* 1974; Muthukkaruppan & Auerbach 1979). As almost all types of corneal injury induce neovascularization, many experimental techniques including chemical cauterization, intrastromal injection, mechanical scraping of the limbal epithelium, intracorneal suture, surgical grafts and most commonly micropockets with implantation of slow release pellets have been employed in the development of corneal angiogenesis assays (Shan & Dewhirst 2006). The original experiments investigating tumour-induced neovascularization in a corneal pocket used tumour fragments implanted in the rabbit corneal pocket (Gimbrone *et al.* 1974), which has since been adapted for use in guinea pigs, rats and mice (Muthukkaruppan & Auerbach 1979; Fournier *et al.* 1981; Hori 1990). Substances used within the pocket have included tumour tissue, tumour cells, tumour cell extracts, other tissues and cells, concentrated conditioned medium, and more recently purified/recombinant cytokines and/or growth factors incorporated into slow release pellets (Conn *et al.* 1980; Fournier *et al.* 1981; Gross *et al.* 1981; D'Amato *et al.* 1994; Lingen *et al.* 1996). It is then possible to treat the animal with a test compound via systemic administration, topical application in eye drops and/or a combined pellet. The route used will depend upon the characteristics, bioavailability and pharmacokinetics of the test compound. Systemic administration has the advantage that it is more clinically relevant and is ideal for use as a preclinical investigation (Volpert *et al.* 1998; Shaw *et al.* 2003).

The vascular response of the cornea is usually achieved by perfusion of the cornea with fluorescent dye or india ink to visualize the blood vessels. The corneas are removed and flattened onto slides, which are imaged at low power on a microscope. While some authors simply score the neovascularization as positive or negative based on certain criteria (Polverini *et al.* 1991), it is possible to quantify different parameters such as vascularized area (often expressed as percentage of the whole cornea), the circumference, vessel length, vascular density and capillary diameter (Shan *et al.* 2001; Sarayba *et al.* 2005). However, it is important to note that some parameters are not always proportional and therefore it is advisable to analyse more than one endpoint to obtain the maximum information from each single experiment. The assay is reliable and readily quantifiable, but is expensive, time consuming and technically demanding with surgery becoming more difficult as the eye size decreases; although use of mice makes the assay cheaper and more adaptable with transgenic or knockout mice readily available, rats are easier to use. As the processes of corneal neovascularization involve numerous interactions within the cornea, it is possible that different pathways of angiogenesis are involved following different experimental techniques, for example, one study showed that $\alpha_v\beta_3$ antagonists inhibited bFGF induced corneal angiogenesis, but not angiogenesis induced by chemical burn (Klotz *et al.* 2000). Moreover, as angiogenesis is not the same in different tissues and the cornea is naturally avascular, there are some questions as to the relevance of using corneal angiogenesis assays for investigating tumour angiogenesis, for example.

Dorsal air sac model

The dorsal air sac model was first proposed by Oikawa and colleagues who showed that the simple implantation of a chamber ring loaded with tumour cells causes angiogenic vessel formation on the murine skin attached to the ring (Oikawa *et al.* 1997). The Millipore chamber ring is 1 cm in diameter and nitrocellulose filters (0.45 μm pore size) are secured on both sides of the ring and the resultant chamber carefully filled with a tumour cell suspension. The dorsal air sac is prepared in anaesthetized mice by lifting the dorsal skin and injecting air (about 8 ml) into the back and then the chamber is implanted through a transverse section cut on the back followed by suturing. Tumour-induced angiogenesis on the skin attached to the chamber ring is readily quantifiable by day 5 after implantation. Although this assay is relatively simple to perform, the surface upon which the chamber is placed must not be irritated, otherwise this may itself induce angiogenesis, thereby masking the vessels formed because of the tumour cells.

Following treatment with a compound of interest, the chamber is carefully detached from the skin exposing the newly formed blood vessels, which are extremely fragile. Angiogenesis is assessed using a dissecting microscope to count vessels or photographs of the skin for quantification of vessel density. However, it is sometimes difficult to distinguish the pre-existing vessels from the new vessels using this simple analysis. More recently, Evans blue has been injected into the mice, which leaks out of the angiogenic vessels thereby accumulating in the interstitial spaces, but is retained within the pre-existing vessels. The accumulation of the dye is then assessed as a semi-quantitative measure of angiogenesis (Yamakawa *et al.* 2004). It is also possible to measure blood volume in the angiogenic skin by determining the amount of ^{51}Cr-labelled erythrocytes circulating in the skin (Funahashi *et al.* 1999) using a gamma counter. However, all these methods have their disadvantages, most especially the difficulty of measuring angiogenesis directly and distinguishing the new blood vessels from the old. Despite that, the simplicity of this method and its adaptability enabling the implantation of tumour cells manipulated to over or under express a certain factor or to load the ring with a single test substance (Hamada *et al.* 2004) make it a useful assay for studying the mechanisms of angiogenesis.

Chamber assays

In vivo imaging of angiogenesis within a transparent chamber enables determination of whether a newly formed blood vessel is perfused and contributing to tissue oxygenation (Vajkoczy *et al.* 1999). It is possible to observe the vasculature using light transillumination (if the tissue is less than 300 μm thick), epi-illumination microscopy or intravital microscopy of various acute preparations where the organs can be made accessible. However, as angiogenesis is a process which lasts over days or weeks, acute preparations are not appropriate for the study of angiogenesis and instead the implantation of a transparent chamber is used. The first transparent chamber preparation was the rabbit ear chamber, which was then adapted for use in mice to quantify structural and functional changes in the neovasculature of tumours (Algire 1943). However, the major drawback of the early studies was the presence of newly formed granulation tissue within the chamber, which influenced the results seen with the tumour implants. Moreover, the rabbit skinfold chamber is expensive for routine use, and a period of 4–6 weeks must pass after surgery before the test substance can be placed within the chamber.

An alternative method, using the dorsal skinfold as a chamber implantation site has been adapted for mice, rats and hamsters (Papenfuss *et al.* 1979; Endrich *et al.* 1980; Lehr *et al.* 1993). In this model, two symmetrical frames are implanted into the dorsal skinfold of the animal, to sandwich the extended double layer of skin; one layer is completely removed exposing the other layer, which is protected by covering with a glass cover slip incorporated into one of the frames (Figure 6; Menger & Lehr 1993). In contrast to the rabbit ear chamber, a recovery period of only 48 h is ideally allowed to overcome any vascular changes caused by the surgery. It is possible to temporarily remove the cover slip for the implantation of tumour cells or transplants into the chamber ready for neovascularization (Laschke *et al.* 2006). However, this preparation has the disadvantage that although the tumours can grow in three dimensions, growth is restricted in one direction by the cover slip. An adaptation of this model is the implantation of mouse metatarsals into the chamber in order to observe revascularization of the bone (Rothenfluh *et al.* 2004). This was further adapted to look at the early stages of tumour metastasis to the bone by observing the homing of fluorescently labelled tumour cells into the metatarsal within the chamber (Reeves *et al.* 2008). The major advantage of these models is that the chambers can be used for repeated intravital microscopic microcirculatory studies in unanaesthetized animals over a time period of 3–4 weeks and the chambers do not provoke angiogenesis or granulation tissue formation (Menger & Lehr 1993). Moreover, analysis of identical blood vessels over time is possible. Trans illumination techniques are used to study microvascular and microcirculatory parameters such as vessel diameter, density and blood flow (Menger *et al.* 2002),

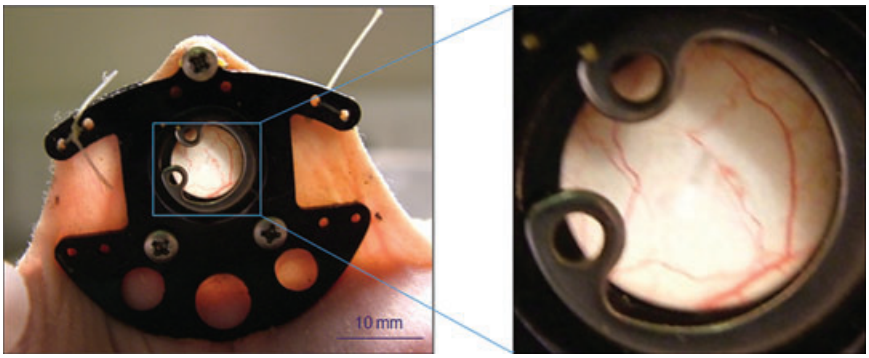

Figure 6 The dorsal skinfold chamber assay. Neovascularization can be observed in the chamber using *in vivo* microscopy.

while epi-illumination fluorescence techniques enable additional *in vivo* analysis of associated parameters including platelet adhesion (Menger & Lehr 1993), vascular permeability (Bingle *et al.* 2006), gene expression (Brown *et al.* 2001), and erythrocyte flow (Reyes-Aldasoro *et al.* 2008). During monitoring, the animal is restrained with the window in the correct position for direct microscopical observation and areas of interest are recorded onto DVD for off-line analysis with an appropriate computer image analysis software package. The ability to measure so many parameters simultaneously and repeatedly in a living conscious animal makes this a very valuable tool for the study of angiogenesis under many conditions including physiological wound healing and tumour growth.

The disadvantage of the skinfold chamber is that it is not an orthotopic site for many of the tumours studied. Therefore, the cranial window preparation was generated to provide an orthotopic brain tumour model (Yuan *et al.* 1994). In this preparation, a bone flap is prepared and the underlying dura removed exposing the brain hemispheres. The tumour specimens, cell suspensions or spheroids can then directly be placed onto the pial surface of either hemisphere and the window sealed with a glass cover slip (Vajkoczy *et al.* 2002). However, as these tumours are not placed intraparenchymally, these are still not truly representative of spontaneous brain tumours.

The final window chamber preparation commonly in use is the bone chamber, which until recently consisted of a hollow titanium screw allowing access to the bone tissue (Winet 1996). However, this did not provide an adequate observation area in mice and has recently been adapted to a sandwich method whereby the mouse femur is sandwiched in the chamber and observed by intravital microscopy (Hansen-Algenstaedt *et al.* 2005). This model has the advantages that the bone within the chamber remains biomechanically active, tumour and metastasis studies can be performed and both the pre-existing and newly growing vessels can be observed.

These chamber assays have been used to study physiological angiogenesis (using implantation of follicles into the dorsal skinfold chamber (Vollmar *et al.* 2001)), tumour angiogenesis (Bingle *et al.* 2006), angiogenesis in endometriosis (by transplanting endometrial tissue into dorsal skinfold chambers (Laschke *et al.* 2005), angiogenesis in wound and bone healing (Vollmar *et al.*, 2002; Hansen-Algenstaedt *et al.* 2005), angiogenesis in ischaemia (Harder *et al.* 2005) and angiogenesis in tissue engineering (Druecke *et al.* 2004). All of the chamber assays have the advantage of allowing the determination of 3D vessel growth in one animal, usually while the animal is conscious, over a period of 1–4 weeks. Therefore, separate groups of mice are not required at each time point thus minimizing the number of animals. Also, the ability to simultaneously study cellular, molecular and functional parameters at each time point makes this an extremely powerful model. However, all chamber assays are invasive and technically demanding requiring sophisticated surgical procedures. Furthermore, quantification of functional parameters is time consuming and requires computer assisted dynamic image analysis systems, and the sites do not represent natural environments for tumour growth and metastasis.

Tumour models

Subcutaneous implantation of tumour cells is one of the easiest approaches for investigating tumour growth and angiogenesis *in vivo* and is widely used (Stribbling *et al.* 2000; Brown *et al.* 2002; Staton *et al.* 2004b). Despite the well documented differences in microenvironment of subcutaneously grown tumours *vs.* tumours grown at an orthotopic site (Keyes *et al.* 2003), this is a valuable model as tumour growth can be easily assessed by measuring tumour dimensions regularly and weighing excised tumours at the end of the study (Stoeltzing *et al.* 2004; Staton *et al.* 2007a). Although angiogenesis cannot be visualized or monitored daily, if the groups are sufficiently powered, subgroups of

randomized animals may be killed sequentially for imaging angiogenesis (Bruns *et al.* 2000). It is generally advised that if subcutaneous tumour experiments have promising results then these results should be confirmed in an orthotopic tumour model where feasible.

As new anti-angiogenic compounds enter clinical trials, the question remains as to whether tumours at several different anatomical sites will respond similarly to the same agent (Jung *et al.* 2002), as organ microenvironment has been shown to play a critical role in angiogenesis (Wilmanns *et al.* 1992). Therefore, it is essential that preclinical models represent the clinical setting, for example, with colorectal cancer cells growing in the colon and metastasizing to the liver (Fidler 1986). Thus a wide variety of orthotopic tumour models have now been developed for most common human cancers. In contrast to subcutaneous models, the injection of the tumour cells is more technically challenging, and measuring or monitoring tumour growth *in vivo* is often not possible, and tumour take is often low. The use of tumour cells transfected to express GFP coupled with imaging of the fluorescence can reduce some of these issues and enable monitoring of tumour growth and the appearance of any metastases over time (Cross *et al.* 2008). However, within these models, metastases may not always occur in the expected organ sites. Despite these difficulties, however, this is a useful model for testing angiogenic drugs.

A major disadvantage of all the tumour models described so far is that tumours are established within a few weeks after cell implantation, whereas human cancer develops over a period of several months or years. However, there are now transgenic mouse models of tumour angiogenesis where animals overexpress targeted oncogenes leading to spontaneous tumour formation over a longer time period. These include the RIP-Tag mice where over expression of the SV40 Tag oncogene is under control of the insulin regulatory region (Hanahan 1985). This is a model of pancreatic island β-cell tumour carcinogenesis, which progresses through a sequence from hyperplastic islets to invasive tumours and eventually to metastasis (Hanahan *et al.* 1996). Similarly, there are two murine models of spontaneous breast cancer currently in use where transgenic mice expressing either the HER2/neu oncogene (Di Carlo *et al.* 1999) or the polyoma middle T oncogene (Lin *et al.* 2003) under control of the mouse mammary tumour virus promoter undergo a multistaged tumour development from atypical hyperplasia to carcinoma *in situ* and invasive carcinoma with histopathological and molecular characteristics closely resembling human breast cancer over a period of several months. We now know the importance of angiogenesis in early stages of carcinogenesis (Menakuru *et al.*, 2008; Staton *et al.* 2007b) and the advantage of such models is that each stage occurs at a particular age of the animal and so studies into angiogenesis in hyperplasia or *in situ* disease can be undertaken.

For the tumours to grow beyond a certain size, they require angiogenesis to obtain oxygen and nutrients and remove waste products, and thus the potential anti-angiogenic effects of a new drug can be tested in all the models described. Moreover, it is possible to establish the effects of a new drug against well vascularized *vs.* poorly vascularized tumours as well as in tumours of vascular origin including haemangiosarcomas (Sato *et al.* 1986) and Kaposi's sarcoma (Ensoli *et al.* 1994). Use of the whole animal system also allows study of the uptake and distribution of a drug candidate as well as its efficacy in the same experiment. Furthermore, it is possible to distinguish between anti-angiogenic (reducing/preventing the formation of new tumour blood vessels) and anti-vascular (damaging the endothelial cell lining of the existing vessels) effects of a test substance. In order to analyse angiogenesis in these models, pathological examination procedures such as histology and immunohistochemistry are used. The histological analyses include measurements of necrosis and morphology of the tumours (H&E staining; Brown *et al.* 2002), thrombosis formation (MSB stain for the detection of fibrin; Brown *et al.* 2002), microvessel density (CD31/CD34 staining with Chalkley grid counting; Staton *et al.* 2007a), actively proliferating endothelial cells (PCNA and/or Endoglin staining; Inoue *et al.* 2003) and apoptosis (TUNEL; Inoue *et al.* 2003). Such measurements are time consuming and require animal sacrifice and therefore preclude ongoing angiogenesis studies in individual, live, tumour bearing animals. Moreover, it must be taken into consideration that in experimental tumour models, the neovasculature is not human and therefore may react differently to the test compounds than that of human vasculature (Goldbrunner *et al.* 2004). Despite these limitations, tumour models are an essential part of current preclinical translational investigations.

Angiomouse

As briefly mentioned in the previous section, Green fluorescent protein (GFP) has been used to study orthotopic tumour angiogenesis, whereby tumours which are transfected to express GFP are placed in their natural microenvironment and therefore mimic clinical behaviour more closely than subcutaneous tumours (Cross *et al.* 2008). These orthotopic tumours give rise to spontaneous metastases that target the same tissues as the original human tumour (Hoffman 1999). In this model, the primary tumour and any metastases are detected by an intense green fluorescence, which can

be imaged by fluorescence stereo microscopy and captured on camera (Cross *et al.* 2008; Figure 7). The non-luminous angiogenic blood vessels appear as sharply defined dark networks against this fluorescent background (Hoffman 2002). From high resolution images of these networks, it is possible to generate quantitative measurements including microvessel density and total vessel length, thus enabling the quantification of angiogenesis, tumour growth and metastasis in real time in a non-invasive manner (Yang *et al.* 2001). The other major advantage is that any differences in angiogenesis between primary and metastatic tumours can be detected, and this is important when considering therapeutic implications (Li *et al.* 2000). The main disadvantage of this method is that whole body imaging is not always sensitive enough with GFP because of light absorption by the surrounding tissues, especially the skin. An improvement on this method has been to use a reversible skinflap, which reduces signal attenuation and increases the signal detection sensitivity (Yang *et al.* 2002). This method involves making an arc shaped incision in the skin of an anaesthetized animal to produce a small flap, which can be repeatedly opened and closed to image the GFP. However, despite this improvement, visualization of very small capillaries is still difficult. Moreover, caution should be taken when interpreting results using GFP-expressing tumours because the fluorescence decreases with reduced oxygenation and is virtually eliminated by anoxia, conditions which exist in both experimental and clinical tumours (Coralli *et al.* 2001).

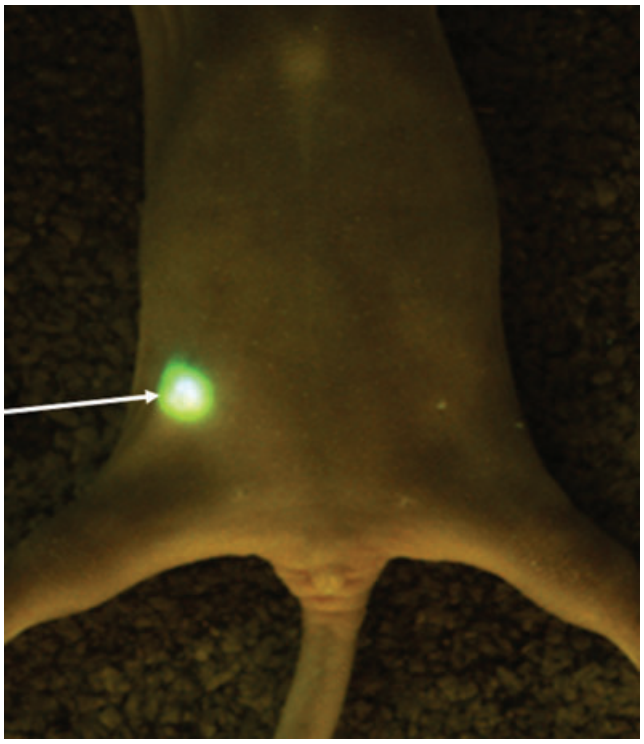

Figure 7 Visualization of tumours growing in orthotopic sites. MDA-MB-436 breast cancer cells transfected to express GFP were injected into the mammary fat pad and can be observed using fluorescent microscopy.

Okabe *et al.* (1997) produced transgenic mice, which expressed GFP in all tissues. These mice have since been crossed with nude mice to produce immunodeficient animals, which also fluoresce green throughout their bodies (Yang *et al.* 2004). These have the advantage that human tumours can be grown and the lack of hair makes imaging easier. By implanting red fluorescent protein (RFP)-expressing tumours into these mice, it is possible to use whole body imaging to generate dual colour images of the early events in tumour angiogenesis, and to visualize and distinguish the components of the host–tumour interaction (Yang *et al.* 2003). The advantage of this method is that there is much greater resolution enabling detection of the very fine vessels; however, for the highest resolution tissue preparation may still be required.

An alternative nude mouse strain where GFP is expressed under the control of the nestin regulatory protein (ND-GFP mouse) has been developed (Amoh *et al.* 2004). In these mice, GFP is expressed in the hair follicles and blood vessels of the skin; so when RFP-tumours are implanted subcutaneously, ND-GFP expression is clearly seen in the proliferating endothelial cells and nascent blood vessels in the growing red fluorescing tumours without the added complication of any other green fluorescing cells (Amoh *et al.* 2005). However, although it is possible to treat tumours and observe differences in blood vessel formation easily over time within this model (Amoh *et al.* 2005), it suffers from the limitations of being a subcutaneous, rather than an orthotopic model. Alternative transgenic mice have been developed where GFP is expressed under the control of either endothelial nitric oxide synthase (eNOS) (Figure 8) or Tie-2, both of which are largely restricted to endothelial cells (Van Haperen *et al.* 2003; Hillen *et al.* 2008). These both have the advantages that all vessels throughout the animal express GFP and therefore orthotopic tumour models are possible. However, both eNOS and Tie-2 expression are under complex regulation and their expression varies under different circumstances [e.g. eNOS expression alters with shear stress (Cheng *et al.* 2005) and Tie-2 expression alters within tumour growth and development (Martin *et al.* 2008)], which may lead to variations in analysis in different regions of the animal, which are not due to variations in angiogenesis.

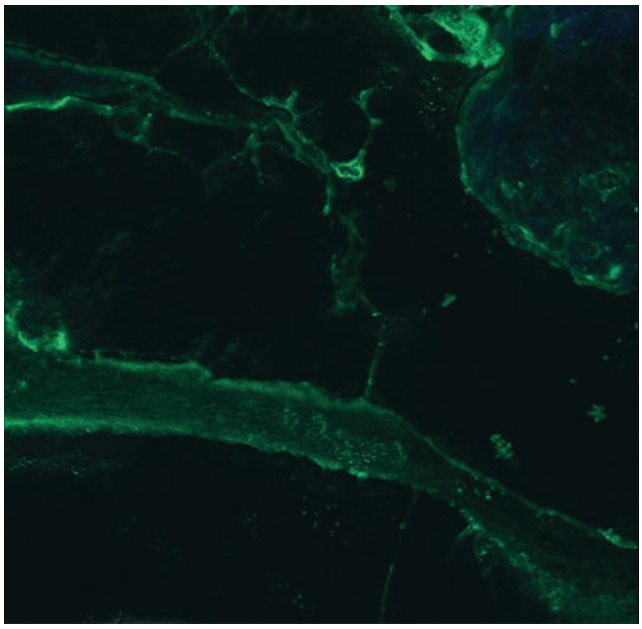

Figure 8 eNOS-GFP vessels. A confocal image of green fluorescent blood vessels, which are clearly visible by virtue of the eNOS expression at the endothelial cell barrier within the eNOS-GFP transgenic mice.

Conclusions

Despite the discovery and development of numerous agents for the treatment of angiogenic diseases (mainly cancer), which have showed dramatic and promising inhibitory effects in preclinical testing *in vitro* and *in vivo*, and in some instances tumour regression, these agents have proved very disappointing in phase II/III clinical trials. This inconsistency of data between preclinical and clinical trials highlights the need for rigorous preclinical testing both *in vitro* and *in vivo* using relevant models and the limitations of each assay being taken into consideration, thus avoiding over interpretation of results.

While *in vitro* assays are often useful for screening potential targets and provide an early validation step in the process of testing a new drug because of their rapid nature and ease of quantification, care must be taken in interpretation. Indeed, the validity of cultured cells as a model of physiological function *in vivo* has been criticized as being too different from the natural cellular environment. However, this is a valuable tool provided the culture conditions are adjusted to mimic closely the *in vivo* situation, and with added validity when endothelial cells are cultured in the presence of supporting cells (smooth muscle cells, mural cells and fibroblasts) and an ECM. Perhaps the selection of appropriate endothelial cells is important to reflect the disease being studied; for example, for psoriasis, human dermal microvascular endothelial cells are used, whereas for breast cancer, breast microvascular endothelial cells are used.

Although the different *ex vivo* cultures in use provide some of the supporting cells and ECM thereby acting as a link between *in vitro* and *in vivo* assays, they are often not from the relevant organ; therefore it is essential that *in vitro* assays be validated by a variety of *in vivo* assays. Moreover, a single *in vivo* model is inadequate to fully investigate the process of angiogenesis as there are variations between species (e.g. zebrafish *vs.* mouse), location/organ sites (e.g. flank *vs.* cornea) and specific microenvironments. The timing, concentration, location and mode of treatment administration are critical in all animal experiments regardless of species. Therefore, the treatment strategy for any *in vivo* model must be able to accommodate limitations in experimental design. Moreover, the concentrations of cytokines required to observe angiogenesis in any *in vivo* assay will vary from the concentrations of angiogenic growth factors used to stimulate endothelial cells in *in vitro* assays. In order to more closely mimic the human clinical situation most *in vivo* experiments are time consuming, allowing for natural angiogenesis to occur (within a spontaneous tumour for example). However, this also makes them costly and prone to variability. Although traditionally *in vivo* experiments were notoriously difficult to analyse, there has been an exponential increase in the sophistication of *in vivo* imaging techniques including the availability of MRI, CT and PCT facilities for scanning small animals, and the advent of confocal and multi-photon microscopy enabling fine structure imaging *in situ*. What is increasingly obvious from all the previous studies is that only an extensive acquisition of experimental *in vivo* data will ultimately allow an adequate interpretation of preclinical studies that potentially translate into promising clinical therapeutic regimens.

It is always difficult to predict the future developments in any area of research. The goals for the development of angiogenesis assays have been well defined for over a decade; improved quantification, rapid assessments, ease of execution, reproducibility and applicability to clinical practice (Jain *et al.* 1997). All these variables remain relevant, but the continued development of sophisticated imaging techniques will improve data acquisition. Other major advances are likely to involve the mechanisms of angiogenesis present in different organs and disease states. Thus, the generation of new and more appropriate disease models may be achieved by transgene protocols, leading to improved diagnostic assays that incorporate angiogenic responses (Auerbach 2006).

In summary, in order to fully interpret the effects of a test substance on the process of angiogenesis, it is still necessary to use multiple *in vitro* assays designed to investigate the different steps in the angiogenesis pathway with either the relevant endothelial cell or multiple endothelial cell types, followed by more than one *in vivo* assay, where the microenvironment will influence angiogenesis and the effect of the test substance. This will enhance the likely success of preclinical studies translating into clinical modalities.

References

Ades E.W., Candal F.J., Swerlick R.A. *et al.* (1992) HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J. Invest. Dermatol.* **99**, 683–690.

Aird W.C. (2003) Endothelial cell heterogeneity. *Crit. Care Med.* **31**, S221–S230.

Akimoto S., Mitsumata M., Sasaguri T., Yoshida Y. (2000) Laminar shear stress inhibits vascular endothelial cell proliferation by inducing cyclin-dependent kinase inhibitor p21(Sdi1/Cip1/Waf1). *Circ. Res.* **86**, 185–190.

Alavi A., Hood J.D., Frausto R., Stupack D.G., Cheresh D.A. (2003) Role of Raf in vascular protection from distinct apoptotic stimuli. *Science* **301**, 94–96.

Albini A., Benelli R., Noonan D.M., Brigati C. (2004) The ''chemoinvasion assay'': a tool to study tumor and endothelial cell invasion of basement membranes. *Int. J. Dev. Biol.* **48**, 563–571.

Albrecht-Buehler G. (1977) Phagokinetic tracks of 3T3 cells: parallels between the orientation of track segments and of cellular structures which contain actin or tubulin. *Cell* **12**, 333–339.

Alby L. & Auerbach R. (1984) Differential adhesion of tumor cells to capillary endothelial cells in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5739.

Alessandri G., Raju F., Gullino P.M. (1983) Mobilization of capillary endothelium in vitro induced by effectors of angiogenesis in vivo. *Cancer Res.* **43**, 1790–1797.

Algire G.H. (1943) An adaptation of the transparent chamber technique to the mouse. *J. Natl. Cancer Inst.* **4**, 1–11.

Amoh Y., Li L., Yang M. *et al.* (2004) Nascent blood vessels in the skin arise from nestin-expressing hair follicle cells. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 13291–13295.

Amoh Y., Li L., Yang M. *et al.* (2005) Hair follicle-derived blood vessels vascularize tumors in skin and are inhibited by doxorubicin. *Cancer Res.* **65**, 2337–2343.

Andrade S.P., Fan T.-P.D., Lewis G.P. (1987) Quantitative *in vivo* studies on angiogenesis in a rat sponge model. *Br. J. Exp. Path.* **68**, 755–766.

Ariano P., Distasi C., Gilardino A., Zamburlin P., Ferraro M. (2005) A simple method to study cellular migration. *J. Neurosci. Methods* **141**, 271–276.

Auerbach R. (2006) An overview of current angiogenesis assays: choice of assay, precautions in interpretation, future requirements and directions. In: *Angiogenesis Assays: A Critical Appraisal of Current Techniques*, pp. 361–374 (eds C.A. Staton, R. Bicknell, C.E. Lewis), Chichester, UK: John Wiley & Sons.

Auerbach R., Arensman R., Kubai L., Folkman J. (1975) Tumor-induced angiogenesis: lack of inhibition by irradiation. *Int. J. Cancer* **15**, 241–245.

Auerbach R., Auerbach W., Polakowski I. (1991) Assays for angiogenesis: a review. *Pharmacol. Ther.* **51**, 1–11.

Auerbach R., Akhtar N., Lewis R.L., Shinners B.L. (2000) Angiogenesis assays: problems and pitfalls. *Cancer Metastasis Rev.* **19**, 167–172.

Auerbach R., Lewis R., Shinners B., Kubai L., Akhtar N. (2003) Angiogenesis assays: a critical overview. *Clin. Chem.* **49**, 32–40.

Ausprunk D.H. & Folkman J. (1976) Vascular injury in transplanted tissues. Fine structural changes in tumor, adult, and embryonic blood vessels. *Virchows Arch. B Cell Pathol.* **21**, 31–44.

Baker J.H.E., Huxham L.A., Kyle A.H., Lam K.K., Minchiton A.I. (2006) Vascular-specific quantification in an *in vivo* Matrigel chamber angiogenesis assay. *Microvasc. Res.* **71**, 69–75.

Belo A.V., Barcelos L.S., Teixeira M.M., Ferreira M.A., Andrade S.P. (2004) Differential effects of antiangiogenic compounds in neovascularization, leukocyte recruitment, VEGF production, and tumour growth in mice. *Cancer Invest.* **22**, 723–729.

Berger A.C., Wang X.Q., Zalatoris A., Cenna J., Watson J.C. (2004) A murine model of ex vivo angiogenesis using aortic disks grown in fibrin clot. *Microvasc. Res.* **68**, 179–187.

Bikfalvi A., Cramer E.M., Tenza D., Tobelem G. (1991) Phenotypic modulations of human umbilical vein endothelial cells and human dermal fibroblasts using two angiogenic assays. *Biol. Cell* **72**, 275–278.

Bingle L., Lewis C.E., Corke K., Reed M.W.R., Brown N.J. (2006) Macrophages potentiate angiogenesis in the dorsal skinfold chamber model. *Br. J. Cancer* **94**, 101–107.

Bishop E.T., Bell G.T., Bloor S., Broom I.J., Hendry N.F., Wheatley D.N. (1999) An in vitro model of angiogenesis: basic features. *Angiogenesis* **3**, 335–344.

Blacher S., Devy L., Burbridge M.F. *et al.* (2001) Improved quantification of angiogenesis in the rat aortic ring assay. *Angiogenesis* **4**, 133–142.

Bollet A.J., Goodwin J.F., Simpson W.F., Anderson D.V. (1958) Mucopolysaccharide, protein and DNA concentration of granulation tissue induced by polyvinyl sponges. *Proc. Soc. Exp. Biol. Med.* **99**, 418–421.

Boyden S. (1962) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. Exp. Med.* **115**, 453–466.

Brown K.J., Maynes S.F., Bezos A., Maguire D.J., Ford M.D., Parish C.R. (1996) A novel in vitro assay for human angiogenesis. *Lab. Invest.* **75**, 539–555.

Brown E.B., Campbell R.B., Tsuzuki Y. *et al.* (2001) *In vivo* measurement of gene expression, angiogenesis and physiological function in tumours using multiphoton laser scanning microscopy. *Nat. Med.* **7**, 864–868.

Brown N.J., Staton C.A., Rodgers G.R., Corke K.P., Underwood J.C.E., Lewis C.E. (2002) Fibrinogen E fragment selectively disrupts the vasculature and inhibits the growth of tumours in a syngeneic murine model. *Br. J. Cancer* **86**, 1813–1816.

Bruns C.J., Harbison M.T., Davis D.W. *et al.* (2000) Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by anti-angiogenic mechanisms. *Clin. Cancer Res.* **6**, 1936–1948.

Cai G., Lian J., Shapiro S.S., Beacham D.A. (2000) Evaluation of endothelial cell migration with a novel in vitro assay system. *Methods Cell Sci.* **22**, 107–114.

Carmeliet P. & Jain R.K. (2000) Angiogenesis in cancer and other diseases. *Nature* **407**, 249–257.

Carson-Walter E.B., Watkins D.N., Nanda A., Vogelstein B., Kinzler K.W., St Croix B. (2001) Cell surface tumour endothelial markers are conserved in mice and humans. *Cancer Res.* **61**, 6649–6655.

Cary L.A., Guan J.L. (1999) Focal adhesion kinase in integrin-mediated signalling. *Front. Biosci.* **4**, 102–113.

Cheng C., van Haperen R., de Waard M. *et al.* (2005) Shear stress affects the intracellular distribution of eNOS: direct demonstration by a novel in vivo technique. *Blood* **106**, 3691–3698.

Chico T.J., Ingham P.W., Crossman D.C. (2008) Modeling cardiovascular disease in the zebrafish. *Trends Cardiovasc. Med.* **18**, 150–155.

Childs S., Chen J.N., Garrity D.M., Fishman M.C. (2002) Patterning of angiogenesis in the zebrafish embryo. *Development* **129**, 973–982.

Cockerill G.W., Varcoe L., Meyer G.T., Vadas M.A., Gamble J.R. (1998) Early events in angiogenesis: cloning an alpha-prolyl 4-hydroxylase-like gene. *Int. J. Oncol.* **13**, 595–600.

Conn H., Berman M., Kenyon K., Langer R., Gage J. (1980) Stromal vascularization prevents corneal ulceration. *Invest. Ophthalmol. Vis. Sci.* **19**, 362–370.

Connolly J.O., Simpson N., Hewlett L., Hall A. (2002) Rac regulates endothelial morphogenesis and capillary assembly. *Mol. Biol. Cell* **13**, 2474–2485.

Coomber B.L. & Gotlieb A.I. (1990) In vitro endothelial wound repair. Interaction of cell migration and proliferation. *Arteriosclerosis* **10**, 215–222.

Coralli C., Cemazar M., Kanthou C., Tozer G.M., Dachs G.U. (2001) Limitations of the reporter green fluorescent protein under simulated tumour conditions. *Cancer Res.* **61**, 4784–4790.

Cross L.M., Cook M.A., Lin S., Chen J.N., Rubinstein A.L. (2003) Rapid analysis of angiogenesis drugs in a live fluorescent zebrafish assay. *Arterioscler. Thromb. Vasc. Biol.* **23**, 911–922.

Cross N.A., Fowles A., Reeves K. *et al.* (2008) Imaging the effects of castration on bone turnover and hormone dependent prostate cancer colonization of bone. *Prostate* **68**, 1707–1714.

Currie P.D. & Ingham P.W. (1996) Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* **382**, 452–455.

D'Amato R.J., Loughnan M.S., Flynn E., Folkman J. (1994) Thalidomide is an inhibitor of angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4082–4085.

Darland D.C., Massingham L.J., Smith S.R., Piek E., Saint-Geniez M., D'Amore P.A. (2003) Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Dev. Biol.* **264**, 275–288.

Davidson J.M., Klagsbrun M., Hill K.E. *et al.* (1985) Accelerated wound repair, cell proliferation and collagen accumulation are produced by a cartilage-derived growth factor. *J. Cell Biol.* **100**, 1219–1227.

Debeir O., Camby I., Kiss R., Van Ham P., Decaestecker C. (2004) A model-based approach for automated in vitro cell tracking and chemotaxis analyses. *Cytometry* **60**, 29–40.

Deckers M., van der Pluijm G., Dooijewaard S. *et al.* (2001) Effect of angiogenic and antiangiogenic compounds on the outgrowth of capillary structures from fetal mouse bone explants. *Lab. Invest.* **81**, 5–15.

Denizot F. & Lang R. (1986) Rapid colormetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89**, 271–277.

Devy L., Blacher S., Grignet-Debrus C. *et al.* (2002) The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *FASEB J.* **16**, 147–154.

Di Carlo E., Diodoro M.G., Boggio K. *et al.* (1999) Analysis of mammary carcinoma onset and progression in HER-2/neu oncogene transgenic mice reveals a lobular origin. *Lab. Invest.* **79**, 1261–1269.

Donovan D., Brown N.J., Bishop E.T., Lewis C.E. (2001) Comparison of three in vitro human 'angiogenesis' assays with capillaries formed in vivo. *Angiogenesis* **4**, 113–121.

Druecke D., Langer S., Lamme E. *et al.* (2004) Neovascularization of poly(ether ester) block-copolymer scaffolds *in vivo*: long-term investigations using intravital fluorescent microscopy. *J. Biomed. Mater. Res. A.* **68**, 10–18.

Edwards R.H., Sarmenta S.S., Hass G.M. (1960) Stimulation of granulation tissue growth by tissue extracts; study by intramuscular wounds in rabbits. *Arch. Path.* **69**, 286–302.

Endrich B., Asaishi K., Goetz A., Messmer K. (1980) Technical report – a new chamber technique for microvascular studies in unanesthetized hamsters. *Res. Exp. Med.* **177**, 125–134.

Ensoli B., Markham P., Kao V. *et al.* (1994) Block of AIDS-Kapsoi's sarcoma (KS) cell growth, angiogenesis and lesion formation in nude mice by antisense oligonucleotide targeting basic fibroblast growth factor. A novel strategy for the therapy of KS. *J. Clin. Invest.* **94**, 1736–1746.

Falk W., Goodwin R.H., Leonard E.J. (1980) A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods* **33**, 239–247.

Fawcett J. (1994) *Molecular Aspects of Tumor Angiogenesis and Metastasis*. D.Phil. thesis, Oxford: University of Oxford.

Ferreira M.A., Barcelos L.S., Campos P.P., Vasconcelos A.C., Teixeira M.M., Andrade S.P. (2004) Sponge-induced angiogenesis and inflammation in PAF receptor-deficient mice (PAFR-KO). *Br. J. Pharmacol.* **141**, 1185–1192.

Fidler I.J. (1986) Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. *Cancer Metastasis Rev.* **5**, 29–49.

Folkman J. (1982) Angiogenesis: initiation and control. *Ann. N. Y. Acad. Sci.* **401**, 212–227.

Fournier G.A., Lutty G.A., Watt S., Fenselau A., Patz A. (1981) A corneal micropocket assay for angiogenesis in the rat eye. *Invest. Ophthalmol. Vis. Sci.* **21**, 351–354.

Fox S.B., Leek R.D., Weekes M.P., Whitehouse R.M., Gatter K.C., Harris A.L. (1995) Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. *J. Pathol.* **177**, 275–283.

Friis T., Kjaer Sorensen B., Engel A.M., Rygaard J., Houen G. (2003) A quantitative ELISA-based co-culture angiogenesis and cell proliferation assay. *APMIS* **111**, 658–668.

Funahashi Y., Wakabayashi T., Semba T., Sonoda J., Kitoh K., Yoshimatsu K. (1999) Establishment of a quantitative mouse dorsal air sac model and its application to evaluate a new angiogenesis inhibitor. *Oncol. Res.* **11**, 319–329.

Gagnon E., Cattaruzzi P., Griffith M. *et al.* (2002) Human vascular endothelial cells with extended life spans: in vitro cell response, protein expression, and angiogenesis. *Angiogenesis* **5**, 21–33.

Gimbrone M.A., Cotran R.S., Leapman S.B., Folkman J. (1974) Tumor growth and neovascularization: an experimental model using the rabbit cornea. *J. Natl. Cancer Inst.* **52**, 413–427.

Go R.S. & Owen W.G. (2003) The rat aortic ring assay for in vitro study of angiogenesis. *Methods Mol. Med.* **85**, 59–64.

Goldbrunner R.H., Bendszus M., Tonn J.C. (2004) Models for angiogenesis in gliomas. *Cancer Treat. Res.* **117**, 115–135.

Gomez D. & Reich N.C. (2003) Stimulation of primary human endothelial cell proliferation by IFN. *J. Immunol.* **170**, 5373–5381.

Goukassian D., Diez-Juan A., Asahara T. *et al.* (2001) Overexpression of p27(Kip1) by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis. *FASEB J.* **15**, 1877–1885.

Grant D.S., Lelkes P.I., Fukuda K., Kleinman H.K. (1991) Intracellular mechanisms involved in basement membrane induced blood vessel differentiation in vitro. *In Vitro Cell. Dev. Biol.* **27A**, 327–336.

Gray C., Packham I.M., Wurmser F. *et al.* (2007) Ischemia is not required for arteriogenesis in zebrafish embryos. *Arterioscler. Thromb. Vasc. Biol.* **27**, 2135–2141.

Gross J., Azizkhan R.G., Biswas C., Bruns R.R., Hsieh D.S., Folkman J. (1981) Inhibition of tumor growth, vascularization, and collagenolysis in the rabbit cornea by medroxyprogesterone. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1176–1180.

Gumkowski F., Kaminska G., Kaminski M., Morrissey L.W., Auerbach R. (1987) Heterogeneity of mouse vascular endothelium: in vitro studies of lymphatic, large blood vessel and microvascular endothelial cells. *Blood Vessels* **24**, 11–23.

Haldi M., Ton C., Seng W.L., McGrath P. (2006) Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. *Angiogenesis* **9**, 136–151.

Hamada Y., Yuki K., Okazaki M. *et al.* (2004) Osteopontin-derived peptide SVVYGLR induces angiogenesis in vivo. *Dent Mater J* **23**, 650–655.

Hanahan D. (1985) Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* **315**, 115–122.

Hanahan D., Christofori G., Naik P., Arbeit J. (1996) Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. *Eur. J. Cancer* **32A**, 2386–2393.

Hansen-Algenstaedt N., Schaefer C., Wolfram L. *et al.* (2005) Femur window–a new approach to microcirculation of living bone *in situ*. *J. Orthop. Res.* **23**, 1073–1082.

Harder Y., Amon M., Schramm R. *et al.* (2005) Heat shock preconditioning reduces ischemic tissue necrosis by heat shock protein (HSP)-32-mediated improvement of the microcirculation rather than induction of ischemic tolerance. *Ann. Surg.* **242**, 869–878.

Hillen F., Kaijzel E.L., Castermans K., oude Egbrink M.G.A., Lowik C.W.G.M., Griffioen A.W. (2008) A transgenic Tie2-GFP athymic mouse model; a tool for vascular biology in xenograft tumours. *Biochem. Biophys. Res. Commun.* **368**, 364–367.

Hirschi K.K. & D'Amore P.A. (1998) In vitro coculture models of vessel formation and function. In: *Vascular Morphogenesis: In Vivo, In Vitro, In Mente*. pp. 132–140 (eds C. Little, V. Mironov, H. Sage), Boston, USA: Birkhauser Publications.

Hirschi K.K., Rohovsky S.A., D'Amore P.A. (1998) PDGF, TGF-β and heterotypic cell-cell interactions mediate the

recruitment and differentiation of 10T1/2 cells to a smooth muscle fate. *J. Cell Biol.* **141**, 805–814.

Hirschi K.K., Rohovsky S.A., Beck L.H., Smith S., D'Amore P.A. (1999) Endothelial cells modulate the proliferation of mural cell precursors via PDGF-BB and heterotypic contact. *Circ. Res.* **84**, 298–305.

Hirschi K.K., Lai L., Belaguli N.S., Dean D., Schwartz R.J., Zimmer W.E. (2001) TGF-β induction of a smooth muscle cell phenotype requires transcriptional and post-transcriptional control of serum response factor. *J. Biol. Chem.* **277**, 6287–6295.

Hirschi K.K., Burt J.M., Hirschi K.D., Dai C. (2003) Gap junction communication mediates TGF-b activation and endothelial-induced mural cell differentiation. *Circ. Res.*. **93**, 429–437.

Hoffman R.M. (1999) Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. *Invest. New Drugs* **17**, 343–359.

Hoffman R.M. (2002) Green fluorescent protein imaging of tumour growth, metastasis, and angiogenesis in mouse models. *Lancet Oncol.* **3**, 546–556.

Holund B., Junker P., Garbarsch C., Christiffersen P., Lorenzen I. (1979) Formation of granulation tissue in subcutaneously implanted sponges in rats. *Acta Pathol. Microbiol. Scand. [A]* **87**, 367–374.

Hori S. (1990) Pathophysiology of intraocular neovascularisation. *Nippon Ganka Gakkai Zasshi Acta Societatis Ophthalmologicae Japonicae* **94**, 1103–1121.

Ilan N., Mahooti S., Madri J.A. (1998) Distinct signal transduction pathways are utilized during the tube formation and survival phases of in vitro angiogenesis. *J. Cell Sci.* **111**, 3621–3631.

Inoue K., Chikazawa M., Fukata S., Yoshikawa C., Shuin T. (2003) Docetaxel enhances the therapeutic effect of the angiogenesis inhibitor TNP-470 (AGM-1470) in metastatic human transitional cell carcinoma. *Clin. Cancer Res.* **9**, 886–899.

Isogai S., Horiguchi M., Weinstein B.M. (2001) The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev. Biol.* **230**, 278–301.

Jackson C.J. & Nguyen M. (1997) Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteases. *Int. J. Biochem. Cell Biol.* **29**, 1167–1177.

Jaffe E.A., Nachman R.L., Becker C.G., Minick C.R. (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52**, 2745–2756.

Jain R.K., Schlenger K., Hoeckel M., Yuan F. (1997) Quantitative angiogenesis assays: progress and problems. *Nat. Med.* **3**, 1203–1208.

Jung S.P., Siegrist B., Wade M.R., Anthony C.T., Woltering E.A. (2001) Inhibition of human angiogenesis with heparin and hydrocortisone. *Angiogenesis* **4**, 175–186.

Jung Y.D., Ahmad S.A., Liu W. *et al.* (2002) The role of the microenvironment and intercellular cross-talk in tumour angiogenesis. *Semin. Cancer Biol.* **12**, 105–112.

Kanzawa S., Endo H., Shioya N. (1993) Improved *in vitro* angiogenesis model by collagen density reduction and the use of type III collagen. *Ann. Plast. Surg.* **30**, 244–251.

Keyes K.A., Mann L., Teicher B., Alvarez E. (2003) Site-dependent angiogenic cytokine production in human tumor xenografts. *Cytokine* **21**, 98–104.

Kleinman H.K., Kleinman H.K., Graf J. *et al.* (1987) Role of basement membranes in cell differentiation. *Ann. N. Y. Acad. Sci.* **513**, 134–145.

Klemke R.L., Leng J., Molander R., Brooks P.C., Vuori K., Cheresh D.A. (1998) CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. *J. Cell Biol.* **140**, 961–972.

Klotz O., Park J.K., Pleyer U., Hartmann C., Baatz H. (2000) Inhibition of corneal neovascularization by alpha(v)-integrin antagonists in the rat. *Graefes Arch. Clin. Exp. Ophthalmol.* **238**, 88–93.

Korff T., Kimmina S., Martiny-Baron G., Augustin H.G. (2001) Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J.* **15**, 447–457.

Kragh M., Hjarnaa P.-J.V., Bramm E., Kristjansen P.E.G., Rygaard J., Binderup L. (2003) *In vivo* chamber angiogenesis assay: an optimized Matrigel plug assay for fast assessment of anti-angiogenic activity. *Int. J. Oncol.* **22**, 305–311.

Kruger E.A., Duray P.H., Tsokos M.G. *et al.* (2000) Endostatin inhibits microvessel formation in the ex vivo rat aortic ring angiogenesis assay. *Biochem. Biophys. Res. Commun.* **268**, 183–191.

Kruger E.A., Duray P.H., Price D.K., Pluda J.M., Figg W.D. (2001) Approaches to preclinical screening of antiangiogenic agents. *Semin. Oncol.* **28**, 570–576.

Lage A.P. & Andrade S.P. (2000) Assessment of angiogenesis and tumor growth in conscious mice by a fluorimetric method. *Microvasc. Res.* **59**, 278–285.

Lampugnani M.G. (1999) Cell migration into a wounded area in vitro. *Methods Mol. Biol.* **96**, 177–182.

Laschke M.W., Elitzsch A., Vollmar B., Menger M.D. (2005) *In vivo* analysis of angiogenesis in endometriosis-like lesions by intravital fluorescence microscopy. *Fertil. Steril.* **84**, 1199–1209.

Laschke M.W., Elitzsch A., Vollmar B., Vajkoczy P., Menger M.D. (2006) Combined inhibition of vascular endothelial growth factor (VEGF), fibroblast growth factor and platelet-derived growth factor, but not inhibition of VEGF alone, effectively suppresses angiogenesis and vessel maturation in endometriotic lesions. *Hum. Reprod.* **21**, 262–268.

Lawley T.J. & Kubota Y. (1989) Induction of morphologic differentiation of endothelial cells in culture. *J. Invest. Dermatol.* **93**, 59S–61S.

Lawson N.D. & Weinstein B.M. (2002) Arteries and veins: making a difference with zebrafish. *Nat. Rev. Genet.* **3**, 674–682.

Lawson N.D., Scheer N., Pham V.N. *et al.* (2001) Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128**, 3675–3683.

Lehr H.A., Leunig M., Menger M.D., Nolte D., Messmer K. (1993) Dorsal skinfold chamber technique for intravital microscopy in nude mice. *Am. J. Pathol.* **143**, 1055–1062.

Leibovich S.J., Polverini P.J., Shepard H.M., Wiseman D.M., Shively V., Nuseir N. (1987) Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. *Nature* **329**, 630–632.

Ley C.D., Olsen M.W.B., Lund E.L., Kristjansen P.E.G. (2004) Angiogenic synergy of bFGF and VEGF is antagonized by Angiopoietin-2 in a modified *in vivo* Matrigel assay. *Microvasc. Res.* **68**, 161–168.

Li C.Y., Shan S., Huang Q. *et al.* (2000) Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models. *J. Natl. Cancer Inst.* **92**, 143–147.

Liekens S., De Clerk E., Neyts J. (2001) Angiogenesis regulators and clinical applications. *Biochem. Pharmacol.* **61**, 253–270.

Lin E.Y., Jones J.G., Li P. *et al.* (2003) Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am. J. Pathol.* **163**, 2113–2126.

Lingen M.W., Polverini P.J., Bouck N.P. (1996) Retinoic acid induces cells cultured from oral squamous cell carcinomas to become anti-angiogenic. *Am. J. Pathol.* **149**, 247–258.

Liu J., Wang X.B., Park D.S., Lisanti M.P. (2002) Caveolin-1 expression enhances endothelial capillary tubule formation. *J. Biol. Chem.* **277**, 10661–10668.

Madri J.A. & Williams S.K. (1983) Capillary endothelial cell cultures: phenotypic modulation by matrix components. *J. Cell Biol.* **97**, 153–165.

Mahadevan V., Hart I.R., Lewis G.P. (1989) Factors influencing blood supply in wound granuloma quantitated by a new *in vivo* technique. *Cancer Res.* **49**, 415–419.

Martin V., Liu D., Fueyo J., Gomez-Manzano C. (2008) Tie2: a journey from normal angiogenesis to cancer and beyond. *Histol. Histopathol.* **23**, 773–780.

McCarthy S.A., Kuzu I., Gatter K.C., Bicknell R. (1991) Heterogeneity of the endothelial cell and its role in organ preference of tumour metastasis. *Trends Pharmacol. Sci.* **12**, 462–467.

Menakuru S.R., Brown N.J., Staton C.A., Reed M.W. (2008) Angiogenesis in pre-malignant conditions. *Br J Cancer* **99**, 1961–1966.

Menger M.D. & Lehr H.A. (1993) Scope and perspectives of intravital microscopy-bridge over from *in vitro* to *in vivo*. *Immunol. Today* **14**, 519–522.

Menger M.D., Laschke M.W., Vollmar B. (2002) Viewing the microcirculation through the window: some twenty years experience with the hamster dorsal skinfold chamber. *Eur. Surg. Res.* **34**, 83–91.

Montesano R., Pepper M.S., Orci L. (1993) Paracrine induction of angiogenesis in vitro by Swiss 3T3 fibroblasts. *J. Cell Sci.* **105**, 1013–1024.

Motoike T., Loughna S., Perens E. *et al.* (2000) Universal GFP reporter for the study of vascular development. *Genesis* **28**, 75–81.

Muller A.M., Hermanns M.I., Cronen C., Kirkpatrick C.J. (2002) Comparative study of adhesion molecule expression in cultured human macro- and microvascular endothelial cells. *Exp. Mol. Pathol.* **73**, 171–180.

Muthukkaruppan V. & Auerbach R. (1979) Angiogenesis in the mouse cornea. *Science* **205**, 1416–1418.

Muthukkaruppan V.R., Shinneers B.L., Lewis R., Park S.J., Baechler B.J., Auerbach R. (2000) The chick embryo aortic arch assay: a new, rapid, quantifiable in vitro method for testing the efficacy of angiogenic and anti-angiogenic factors in a three-dimensional, serum-free organ culture system. *Proc. Am. Assoc. Cancer Res.* **41**, 65.

Neckers L.M., Funkhouser W.K., Trepel J.B., Cossman J., Gratzner H.G. (1995) Significant non-s-phase DNA synthesis visualised by flow cytometry in activated and in malignant human lymphoid cells. *Exp. Cell Res.* **156**, 429–438.

Nehls V. & Drenckhahn D. (1995) A novel, microcarrier-based in vitro assay for rapid and reliable quantification of three-dimensional cell migration and angiogenesis. *Microvasc. Res.* **50**, 311–322.

Nicosia R.F. & Ottinetti A. (1990) Modulation of microvascular growth and morphogenesis by reconstituted basement membrane gel in three-dimensional cultures of rat aorta: a comparative study of angiogenesis in matrigel, collagen, fibrin, and plasma clot. *In Vitro Cell Dev. Biol.* **26**, 119–128.

Nicosia R.F., Lin Y.J., Hazelton D., Qian X. (1997) Endogenous regulation of angiogenesis in the rat aorta model. Role of vascular endothelial growth factor. *Am. J. Pathol.* **151**, 1379–1386.

Nicosia R.F., Zhu W.H., Fogel E., Howson K.M., Aplin A.C. (2005) A new ex vivo model to study venous angiogenesis and arterio-venous anastomosis formation. *J. Vasc. Res.* **42**, 111–119.

Nisato R.E., Harrison J.A., Buser R. *et al.* (2004) Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span. *Am. J. Pathol.* **165**, 11–24.

Obeso J.L. & Auerbach R. (1984) A new microtechnique for quantitating cell movement in vitro using polystyrene bead monolayers. *J. Immunol. Methods* **70**, 141–152.

Oikawa T., Sasaki M., Inose M. *et al.* (1997) Effects of cytogenin, a novel microbial product, on embryonic and tumor cell-induced angiogenic responses in vivo. *Anticancer Res.* **17**, 1881–1886.

Okabe M., Ikawa M., Kominami K., Nakanishi T., Nishimune Y. (1997) 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313–319.

Papenfuss H.D., Gross J.F., Intaglietta M., Treese F.A. (1979) A transparent access chamber for the rat dorsal skin fold. *Microvasc. Res.* **18**, 311–318.

Parent C.A. & Devreotes P.N. (1999) A cell's sense of direction. *Science* **284**, 765–770.

Passaniti A., Taylor A.M., Pili R. *et al.* (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab. Invest.* **67**, 519–528.

Paulini K., Korner B., Beneke G., Endres R. (1974) A quantitative study of the growth of connective tissue: investigations on polyester-polyurethane sponges. *Connect. Tissue Res.* **2**, 257–264.

Pepper M.S., Belin D., Montesano R., Orci L., Vassalli J.D. (1990) Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. *J. Cell Biol.* **111**, 743–755.

Plunkett M.L. & Hailey J.A. (1990) An *in vivo* quantitative angiogenesis model using tumor cells entrapped in alginate. *Lab. Invest.* **62**, 510–517.

Polverini P.J., Bouck N.P., Rastinejad F. (1991) Assay and purification of naturally occurring inhibitor of angiogenesis. *Methods Enzymol.* **198**, 440–450.

Reeves K.J., van der Pluijm G., Cecchini M.G., Eaton C.L., Hamdy F.C., Brown N.J. (2008) The dorsal skinfold chamber (DSC): a new model to study prostate cancer metastasis in vivo. *J. Vasc. Res.* **45**, 63.

Reyes-Aldasoro C.C., Akerman S., Tozer G.M. (2008) Measuring the velocity of fluorescently labelled red blood cells with a keyhole tracking algorithm. *J. Microsc.* **229**, 162–173.

Rothenfluh D.A., Demhartner T.J., Fraitzl C.R., Cecchini M.G., Ganz R., Leunig M. (2004) Potential role of pre-existing blood vessels for vascularisation and mineralization of osteochondral grafts. *Acta Orthop. Scand.* **75**, 359–365.

Rubinstein A.L. (2003) Zebrafish: from disease modeling to drug discovery. *Curr. Opin. Drug Discov. Devel.* **6**, 218–223.

Sakuda H., Nakashima Y., Kuriyama S., Sueishi K. (1992) Media conditioned by smooth muscle cells cultured in a variety of hypoxic environments stimulates in vitro angiogenesis. A relationship to transforming growth factor-beta 1. *Am. J. Pathol.* **141**, 1507–1516.

Salvatore J.E., Gilmer W.S., Kashgarian M., Barbee W.R. (1961) An experimental study of the influence of pore size of implanted polyurethane sponges upon subsequent tissue formation. *Surg. Gynecol. Obstet.* **112**, 463–468.

Santiago A. & Erickson C.A. (2002) Ephrin-B ligands play a dual role in the control of neural crest cell migration. *Development* **129**, 3621–3632.

Sanz L., Pascual M., Munoz A., Gonzalez M.A., Salvador C.H., Alvarez-Vallina L. (2002) Development of a computer-assisted high-throughput screening platform for anti-angiogenic testing. *Microvasc. Res.* **63**, 335–339.

Sarayba M.A., Li L., Tungsiripat T. *et al.* (2005) Inhibition of corneal neovascularization by a peroxisome proliferator-activated receptor-gamma ligand. *Exp. Eye Res.* **80**, 435–442.

Sato N., Sato T., Takahashi S., Kikuchi K. (1986) Establishment of murine endothelial cell lines that develop angiosarcomas in vivo: brief demonstration of a proposed animal model for Kaposi's sarcoma. *Cancer Res.* **46**, 362–366.

Schilling J.A., Joel W., Shurley H.M. (1959) Wound healing: a comparative study of the histochemical changes in granulation tissue contained stainless steel wire mesh and polyvinyl cylinders. *Surgery* **46**, 702–710.

Segura I., Serrano A., De Buitrago G.G. *et al.* (2002) Inhibition of programmed cell death impairs in vitro vascular-like structure formation and reduces in vivo angiogenesis. *FASEB J.* **16**, 833–841.

Shan S. & Dewhirst M.W. (2006) Corneal angiogenesis assay. In: *Angiogenesis Assays: A Critical Appraisal of Current Techniques*. pp. 203–222 (eds C.A. Staton, R. Bicknell, C.E. Lewis), Chichester, UK: John Wiley & Sons.

Shan S., Lockhart A.C., Saito W.Y., Knapp A.M., Laderoute K.R., Dewhirst M.W. (2001) The novel tubulin-binding drug bto-956 inhibits r3230ac mammary carcinoma growth and angiogenesis in fischer 344 rats. *Clin. Cancer Res.* **7**, 2590–2596.

Shao R. & Guo X. (2004) Human microvascular endothelial cells immortalized with human telomerase catalytic protein: a model for the study of in vitro angiogenesis. *Biochem. Biophys. Res. Commun.* **321**, 788–794.

Shaw J.P., Chuang N., Yee H., Shamamian P. (2003) Polymorphonuclear neutrophils promote rfgf-2-induced angiogenesis in vivo. *J. Surg. Res.* **109**, 37–42.

Smith J.T., Tomfohr J.K., Wells M.C., Beebe T.P., Kepler T.B., Reichert W.M. (2004) Measurement of cell migration on surface-bound fibronectin gradients. *Langmuir* **20**, 8279–8286.

Solowiej A., Biswas P., Graesser D., Madri J.A. (2003) Lack of platelet endothelial cell adhesion molecule-1 attenuates foreign body inflammation because of decreased angiogenesis. *Am. J. Pathol.* **162**, 953–962.

Sprugel K.M., McPherson J.M., Clowes A.W., Ross R. (1987) Effects of growth factors *in vivo*. I. Cell ingrowth into porus subcutaneous chambers. *Am. J. Pathol.* **129**, 601–613.

Staton C.A., Brown N.J., Rodgers G.R. *et al.* (2004a) Alphastatin, a 24-amino acid fragment of human fibrinogen, is a potent new inhibitor of activated endothelial cells in vitro and in vivo. *Blood* **103**, 601–606.

Staton C.A., Stribbling S.M., Tazzyman S., Hughes R., Brown N.J., Lewis C.E. (2004b) Current methods for assaying angiogenesis in vitro and in vivo. *Int. J. Exp. Pathol.* **85**, 233–248.

Staton C.A., Stribbling S.M., Garcia-Echeverria C. *et al.* (2007a) Identification of key residues involved in mediating the in vivo anti-tumour/anti-endothelial activity of Alphastatin. *J. Thromb. Haemost.* **5**, 846–854.

Straton C.A., Chetwood A.S., Cameron I.C., Cross S.S., Brown N.J., Reed M.W. (2007b) The angiogenic switch occurs at the adenoma stage of the adenoma-carcinoma sequence in colorectal cancer. *Gut.* **56**, 1426–1432.

Stiffey-Wilusz J., Boice J.A., Ronan J., Fletcher A.M., Anderson M.S. (2001) An ex vivo angiogenesis assay utilizing commercial porcine carotid artery: modification of the rat aortic ring assay. *Angiogenesis* **4**, 3–9.

Stoeltzing O., McCarty M.F., Wey J.S. *et al.* (2004) Role of hypoxia-inducible factor 1alpha in gastric cancer cell growth, angiogenesis, and vessel maturation. *J. Natl. Cancer Inst.* **96**, 946–956.

Stoletov K., Montel V., Lester R.D., Gonias S.L., Klemke R. (2007) High resolution imaging of the dynamic tumour cell-vascular interface in transparent zebrafish. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 17406–17411.

Stribbling S.M., Friedlos F., Martin J. *et al.* (2000) Regressions of established breast carcinoma xenografts by carboxypeptidase G2 suicide gene therapy and the produrg CMDA are due to a bystander effect. *Hum. Gene Ther.* **11**, 285–292.

Sun X.T., Ding Y.T., Yan X.G. *et al.* (2004) Angiogenic synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in an in vitro quantitative microcarrier-based three-dimensional fibrin angiogenesis system. *World J. Gastroenterol.* **10**, 2524–2528.

Taraboletti G., Roberts D., Liotta L.A., Giavazzi R. (1990) Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. *J. Cell Biol.*, **111**, 765–772.

Vajkoczy P., Menger M.D., Vollmar B. *et al.* (1999) Inhibition of tumour growth, angiogenesis, and microcirculation by the novel Flk-1 inhibitor SU5416 as assessed by intravital multi-fluorescence videomicroscopy. *Neoplasia* **1**, 31–41.

Vajkoczy P., Farhadi M., Gaumann A. *et al.* (2002) Microtumour growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J. Clin. Invest.* **109**, 777–785.

Van Haperen R., Cheng C., Mees B.M.E. *et al.* (2003) Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice. *Am. J. Pathol.* **163**, 1677–1686.

Vogel H.B. & Berry R.G. (1975) Chorioallantoic membrane heterotransplantation of human brain tumors. *Int. J. Cancer* **15**, 401–408.

Vollmar B., El-Gibaly A.M., Scheuer C., Strik M.W., Bruch H.P., Menger M.D. (2002) Acceleration of cutaneous wound healing by transient p53 inhibition. *Lab. Invest.* **82**, 1063–1071.

Vollmar B., Laschke M.W., Rohan R., Koenig J., Menger M.D. (2001) *In vivo* imaging of physiological angiogenesis from immature to preovulatory ovarian follicles. *Am. J. Pathol.* **159**, 1661–1670.

Volpert O.V., Fong T., Koch A.E. *et al.* (1998) Inhibition of angiogenesis by interleukin 4. *J. Exp. Med.* **188**, 1039–1046.

Weinstein B.M., Stemple D.L., Driever W., Fishman M.C. (1995) Gridlock, a localized heritable vascular patterning defect in the zebrafish. *Nat. Med.* **1**, 1143–1147.

Wemme H., Pfeifer S., Heck R., Muller-Quernheim J. (1992) Measurement of lymphocyte proliferation: critical analysis of radioactive and photometric methods. *Immunobiology* **185**, 78–89.

Wilmanns C., Fan D., O'Brian C.A., Bucana C.D., Fidler I.J. (1992) Orthotopic and ectopic organ environments differentially influence the sensitivity of murine colon carcinoma cells to doxorubicin and 5-fluorouracil. *Int. J. Cancer* **52**, 98–104.

Winet H. (1996) The role of microvasculature in normal and perturbed bone healing as revealed by intravital microscopy. *Bone* **19**, 39S–57S.

Wong M.K. & Gotlieb A.I. (1984) In vitro re-endothelialization of a single-cell wound. Role of microfilament bundles in rapid lamellipodia-mediated wound closure. *Lab. Invest.* **51**, 75–81.

Wong M.K. & Gotlieb A.I. (1988) The reorganization of microfilaments, centrosomes, and microtubules during in vitro small wound reendothelialization. *J. Cell Biol.* **107**, 1777–1783.

Yamakawa S., Asai T., Uchida T., Matsukawa M., Akizawa T., Oku N. (2004) (-)-Epigallocatechin gallate inhibits membrane-type 1 matrix metalloproteinase, MT1-MMP, and tumor angiogenesis. *Cancer Lett.* **210**, 47–55.

Yang M., Baranov E., Li X.-M. *et al.* (2001) Whole-body and intravital optical imaging of angiogenesis in orthotopically implanted tumors. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2616–2621.

Yang M., Baranov E., Wang J.-W. *et al.* (2002) Direct external imaging of nascent cancer, tumor progression, angiogenesis, and metastasis on internal organs in the fluorescent orthotopic model. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3824–3829.

Yang M., Li L., Jiang P., Moossa A.R., Penman S., Hoffman R.M. (2003) Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and

stromal cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 14259–14262.

Yang M., Reynoso J., Jiang P., Li L., Moossa A.R., Hoffman R.M. (2004) Transgenic nude mouse with ubiquitous green fluorescent protein expression as a host for human tumors. *Cancer Res.* **64**, 8651–8656.

Yu C.H., Wu J., Su Y.F. *et al.* (2004) Anti-proliferation effect of 3-amino-2-imino-3,4-dihydro-2H-1,3-benzothiazin-4-one (BJ-601) on human vascular endothelial cells: G0/G1 p21-associated cell cycle arrest. *Biochem. Pharmacol.* **67**, 1907–1916.

Yuan F., Salehi H.A., Boucher Y., Vasthare U.S., Tuma R.F., Jain R.K. (1994) Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial windows. *Cancer Res.* **54**, 4564–4568.

Zetter B.R. (1987) Assay of capillary endothelial cell migration. *Methods Enzymol.* **147**, 135–144.

Zetter B.R. (1988) Endothelial heterogeneity: influence of vessel size, organ localizaton, and species specificity on the properties of cultured endothelial cells. In: *Endothelial Cells*, **Vol. 2** pp. 64–79 (ed U.S. Ryan), Boca Raton, USA: CRC Press.

Zheng L., Ling P., Wang Z. *et al.* (2007) A novel polypeptide from shark cartilage with potent anti-angiogenic activity. *Cancer Biol. Ther.* **6**, 775–780.

Zhong T.P., Childs S., Leu J.P., Fishman M.C. (2001) Gridlock signalling pathway fashions the first embryonic artery. *Nature* **414**, 216–220.

Zhu W.H. & Nicosia R.F. (2002) The thin prep rat aortic ring assay: a modified method for the characterization of angiogenesis in whole mounts. *Angiogenesis* **5**, 81–86.

Zijlstra A., Seandel M., Kupriyanova T.A. *et al.* (2006) Proangiogenic role of neutrophil-like inflammatory heterophils during neovascularization induced by growth factors and human tumor cells. *Blood* **107**, 317–327.

Zimrin A.B., Villeponteau B., Maciag T. (1995) Models of in vitro angiogenesis: endothelial cell differentiation on fibrin but not matrigel is transcriptionally dependent. *Biochem. Biophys. Res. Commun.* **213**, 630–638.

